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THE IN VITRO ACTION OF RAT IMMUNE SERUM ON THE LARVAE OF *TAENIA TAENIAEFORMIS*

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Studies of *Cysticercus fasciolaris*, the larval stage of *Taenia taeniaeformis*, in rats by Miller (1932 and 1934), Miller and Gardiner (1932 and 1934) and Campbell (1936 and 1938a, b and c) have clearly demonstrated that acquired immunity is dependent upon antibodies. Furthermore, specific immune serums acting chiefly as precipitins are deleterious in vitro to certain parasites such as *Nippostrongylus muris* (Sarles, 1938), *Ancylostoma caninum* (Otto, 1939), *Trichinella spiralis* (Oliver-González, 1940) *Trichosomoides crassicauda* (Smith, 1946), *Schistosoma* (Papirmeister and Bang, 1948) and *Ascaridia galli* (Sadun, 1949). A similar in vitro study of *C. fasciolaris* seemed in order. It should be noted, however, that anatomical structures of the foregoing parasites are different from those of *C. fasciolaris* and may lead to different reactions. Indeed, Reuling (1919) described only a desquamation of body cells when glochidia, the larval form of fresh water mussels, were placed in immune serum. Other tests indicated only a weak precipitin in the blood.

MATERIALS AND METHODS

Ova of *T. taeniaeformis* were obtained by concentrating the feces of a few infected cats.

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Male rats were bought from a commercial firm. Since an occasional rat was infected upon arrival, rats were necropsied at the termination of critical experiments, and data from the rare infected ones were discarded. Since only young rats were used for critical experiments, accidental infections could be easily recognized. Rats were infected by passing ova into their stomach via a short rubber tube. They were bled by cardiac puncture under ether and were injected into the femoral vein or intraperitoneally. For artificial immunization the method of Campbell (1938b) was used. This procedure involved 9 equal intraperitoneal injections of a total of 0.18 g of larval material with a 3-day interval between injections. The rats were bled 5 days after the last injection. Fresh or lyophilized material was used.

Onchospheres or the hexacanth embryos were obtained as follows: Ova were treated with trypsin (1% in 1% $NaHCO_3$) at body temperature and were then placed in physiological saline in an electric stirrer for a few minutes. The liberated onchospheres were then picked up under a Greenough binocular microscope.

Cysticerci were obtained by cutting them out of the livers of rats with a fine pair of scissors and placing them in physiological saline. They were always used on the same day they were obtained.

Hanging drop in vitro preparations were usually made on glass plates containing 9 concavities (3 rows of 3). The material was sealed in the concavities with a vaselined cover glass. Large cysts were placed directly in the concavity or in Wassermann tubes. They were kept at 37 C in the incubator or, when continuously observed, were placed on a warm stage on the microscope.

Immune serums were absorbed with lyophilized cysts, ground fresh larval cysts, according to the method of Campbell, or with living cysts. The finely ground larval paste was added to serum to make a 2 to 4% suspension, was stirred in a water bath at 40 C for an hour, was stored in the refrigerator overnight and centrifuged to remove any insoluble material. As a rule, 10 living cysts, 20 to 30 days old, were incubated in 1 cc of serum overnight at 37 C. When observed the next morning, most of them were collapsed and had sunk to the bottom of the tube. In case all had collapsed, the serum was reabsorbed with fresh cysts until all or some remained normal in appearance.

TABLE 1.—*In vitro action of fresh and inactivated immune and normal serums with and without complement on 22- to 26-day old cysticerci.*

Serum in ml			Undiluted complement	Individual effect* on two cysticerci when incubated at 37 C for: ½ hour		4 hours		Overnight		Effect of: Inactivation	Adding complement	Frequency occurrence
Immune rat 1, infected 1 month	Fresh	0.3		++	++	+++	+++	+++	+++	No appreciable change	No increase in activity but a speeding up of the reaction	24%
		0.3	0.01	++	++	+++	+++	+++	+++			
	Inact.*	0.3		—	—	++	++	+++	+++			
		0.3	0.01	—	—	+++	+++	+++	+++			
Immune rat 2, infected 6 months	Fresh	0.3		—	—	+++	+++	+++	+++	Loss of activity	Partial restoration of loss	35%
		0.3	0.01	—	—	+++	+++	+++	+++			
	Inact.	0.3		—	—	—	—	—	—			
		0.3	0.01	—	—	++	+	+++	—			
Pooled immune rat serum, infected 25 to 60 days	Fresh	0.3		—	—	+++	++	+++	+++	Loss of activity	No restoration of loss	29%
		0.3	0.01	—	—	+++	+++	+++	+++			
	Inact.	0.3		—	—	—	—	—	—			
		0.3	0.01	—	—	—	—	—	—			
Immune rat 3, infected 40 days	Fresh	0.3		+	+	+++	+++	+++	+++	Partial loss of activity	Difference in activity on the two cysticerci	12%
		0.3	0.01	+	+	+++	+++	+++	+++			
	Inact.	0.3		+	—	+	—	+	—			
		0.3	0.01	+	—	++	—	+++	—			
Normal rat	Fresh	0.3		++	—	+++	—	++	—	Loss of activity	No restoration of loss	
		0.3	0.01	++	—	+++	—	+++	—			
	Inact.	0.3		—	—	—	—	—	—			
		0.3	0.01	—	—	—	—	—	—			
Normal guinea pig	Fresh	0.3		++	++	+++	+++	+++	+++	Loss of activity	No restoration of loss	
		0.3	0.01	+	+	+++	+++	+++	+++			
	Inact.	0.3		—	—	—	—	—	—			
		0.3	0.01	—	—	—	—	—	—			
Physiological saline				+	++	++	++	+++	++			

* +++, very shrunken and collapsed; ++, much shrunken; +, somewhat shrunken or smaller; —, normal. Inact., serum heated at 56 C for one hour.

EXPERIMENTAL RESULTS

Action of immune serum on onchospheres

Miller (1932 and 1934), Miller and Gardiner (1934) and Campbell (1936 and 1938a, b and c) observed that when onchospheres are fed to immune or passively immune rats, many are prevented from getting into the liver, the site of infection, and those which get to the liver may die without developing further. Campbell called the former early immunity and the latter late immunity. The following experiments were accordingly devised to ascertain whether an activity of immune serum can be demonstrated in vitro on the onchospheres.

Vaseline-sealed hanging drop preparations were made containing 10 ova previously treated with trypsin but still intact and 7-, 15-, 21- or 28-day pooled immune serum from the same rats. While under observation, the cover glass was tapped lightly until some of the onchospheres were exposed by the cracking of the shell of the ova. No reaction was observed between the serums and the onchospheres or discharged substances during one hour. Similar results were obtained when the preparations were kept on a warm stage, when the ova were not treated with trypsin, when the preparations were maintained at 37 C for 6 days or when they contained normal serum. The immune serum had a precipitin titer from 1:200 to 1:800, i.e., antigen dilution in terms of dry weight of cysticerci when tested by the interphase ring test. In addition, 1 ml of the immune serum per 100 g body weight protected 130 to 140 g rats, while control rats receiving physiological saline or normal serum showed 294 and 214 cysts, respectively. This experiment indicated that immune serum from rats infected 4 weeks or less does not cause any precipitates or produce noticeably deleterious effects on the onchospheres in vitro although it completely protects rats from the development of a heavy dose of onchospheres.

Action of normal serum on cysticerci

A confusing finding during early experiments was that many fresh individual or pooled normal rat serums possessed marked cysticercocidal effects when observed in vaseline-sealed hanging drop preparations (normal rat serum, table 1). The reactions were indistinguishable from those observed with immune serums, i.e., they began with the collapsing of the bladder, proceeded with the desquamation of the bladder wall, and ended with the formation of what seemed to be slight precipitate. An extensive study failed to give any indication that the property in fresh normal rat serums was due to accidental infection of the rats. These properties of normal serums were lost after heating for 30 minutes at 56 C and especially when inactivated for one hour. They were also gradually lost by aging in the refrigerator and had largely disappeared after a week. Activity was not restored by fresh guinea pig serum or dilutions of 1:30 although concentrated fresh guinea pig serum acted as did normal rat serum (normal guinea pig serum, table 1).

Physiological saline was also found to be cysticercocidal (table 1). Hence, in the in vitro tests, a minimum of saline was used. As saline was replaced by any negative serum, its reactivity disappeared. This protective action of serum colloids is reminiscent of the situation in the pathogenic trypanosomes (Yorke et al, 1929).

Action of immune serum on cysticerci

Vaseline-sealed preparations were made using, in each one, two 26-day-old cysticerci and a test serum from various individual rats or pooled serum from rats infected 15 to 66 days. In these

tests the cysticerci would usually become collapsed and shrunken with cells protruding from the bladder wall. Scanty precipitates often occurred here and there around the shrunken parasite, but it was impossible to tell if these were immune precipitates because similar material appeared in saline and normal serum. The cysticerci eventually died. The effectiveness of some but not of all the immune serums was often partially lost when inactivated for one half or one hour at 56 C and was restored to varying extents when complement in a final dilution of 1:30 was added.

TABLE 2.—*In vitro action of inactivated immune serums from rats infected for 1 to 6 weeks on 13- to 49-day old cysticerci.*

Immune serum from rats infected:	Individual effect* after 4 and 20 hours incubation at 37 C on cysticerci (3 in each test) of the following age:											
	13 days			20 days			30 days			49 days		
7 days	++ +++	+ +++	– +	+++ +++	+++ +++	+++ +++	+ ++	– ++	– ++	– –	– –	–*
14 days	++ +++	++ +++	– +	+++ +++	++ +++	+ +++	+ +++	+ +++	– ++	– –	– –	– –
21 days	++ +++	++ +++	+ ++	+++ +++	++ +++	++ +++	+ +++	– ++	– ++	– –	– –	– –
28 days	+++ +++	+++ +++	– +	+++ +++	+++ +++	++ +++	+ +++	+ +++	+ +++	– ++	– ++	– ++
35 days	++ ++	++ ++	– ++	+++ +++	+++ +++	++ +++	++ +++	+ +++	– +++	– ++	– ++	– +
42 days	+++ +++	+++ +++	– +	+++ +++	++ +++	+ +++	– +++	– ++	– ++	– –	– –	– –
Normal rat serum	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
Lyophilized 14 days				– –	– –	– –						
21 days				– ++	– –	– –						
28 days				– +	– –	– –						

* 4-hour reading is first line; 20-hour reading is second line.

In spite of an extensive study involving 37 individual and 35 pooled immune serums, variable results were obtained with heat or age inactivation and complementation with fresh guinea pig serum. In some cases, heating for 30 minutes at 56 C did not appreciably lower the cysticercocidal property nor did the addition of complement increase its activity although it did cause the reaction to reach an end point more rapidly (rat serum 1, table 1). In other cases, heat inactivation reduced the cysticercocidal property slightly or moderately, and the addition of complement partially or completely restored the lost activity (rat serum 3, table 1). In still others, heating the serum more or less completely inactivated the serum and the addition of complement partially restored the activity (one of the cysticerci tested with immune rat serum from rat 2 in table 1).

A few individual serums and 6 pooled supposedly immune serums lost their activity after heating to 56 C for 30 minutes and did not regain their activity after the addition of fresh complement (the second of the cysticerci tested with immune serum from rat 3 in table 1). It is probable that such serums possessed little immune principle and that the activity was that of most fresh normal serums.

Various immune serums inactivated for one half or one hour at 56 C were tested on cysticerci of various ages.

The results are recorded in table 2. The parasites were examined at ½, 1, 1½, 2½, and 4 hours and on the following morning. As any effect was usually progressive, only the last reading (about 16 hours incubation) is recorded in the table. A comparison of the results listed in table 2 seems to indicate that cysts become more resistant to the action of immune serum as they grow older. This effect is apparently the case also in vivo in immune animals since routine autopsy examinations have shown many more younger dead cysts than older ones. The basis for the two effects, however, may be different. Thus, Bullock and Curtis (1924) showed that old cysts may be successfully walled off by the host reaction tissue and therefore are not easily accessible to immune serum. On the other hand, as a result of growth, more metabolic products may be secreted which quickly neutralize the active principle of in vitro immune serum or the bladder wall of older cysts may be less permeable. For routine in vitro work, cysts between 15 and 30 days old seem most suitable. The results in table 2 also indicate that the cysticercocidal substance is present in 7-day immune serum. In one instance, it was present as early as the 4th day. It also may get stronger through the 35th day and weaker later as indicated by a stronger reaction against the 49-day-old larvae at 4 and 5 weeks than before or after.

Tests on immune serums from rats with various grades of infection indicated that the injurious effect was weak in lightly infected rats, i.e., when only 30 to 40 tiny cysts were found and many were dead. Such rats unquestionably had a high resistance to the infection but developed little or no cysticercocidal substance. On the other hand, the injurious effect was also weak in highly infected rats, probably because such rats were poor antibody producers or the antibody may have been absorbed by the parasites. The conclusion may be tentatively reached that the cysticercocidal substance is strongest in the serums of moderately infected rats.

Further analysis indicated that the potency of the cysticercocidal substance is greatly reduced by lyophilizing (table 2) whereas the in vivo protective property of immune serum usually is not. The cyticercocidal substance, therefore, does not account for the protective property of immune serum. It is also different from complement since complement is well preserved in the lyopholized state.

The cysticercocidal substance was not found in artificially immunized rats even though serum for such rats may have a precipitin titer as high as 1:3200. Immune serum, after overnight contact with an excess number of living cysticerci or finely ground fresh larvae, lost its anticysticercal power. In other words, the property was absorbed by living or freshly ground cysts.

Functional role of the cysticercocidal substance

The presence of a cysticercocidal substance in serums from rats infected with the larval stage of *T. taeniaeformis* is well established by the preceding experiments. A preliminary study of its function indicates that it does not prevent infection. Thus, in table 3, the average number of parasites in groups 1 and 3 which received immune serum absorbed with living cysts was approximately the same as in groups 2 and 4 which received untreated immune serum. Although by the method employed (see Materials and Methods), the cysticercocidal substance may not have been completely neutralized or removed, it is to be noted that absorption was not considered satisfactory unless some cysts re-

mained normal overnight in the absorbed serum. Moreover, the protective result in group 5 which received lyophilized immune serum is significant. Thus, the protective property remained in the lyophilized serum while the cysticercocidal property was definitely weakened as seen in table 2. In addition, two batches of pooled immune serum which completely protected rats against a heavy dose of ova produced no in vitro effect on the larvae. The cysticercocidal substance, therefore, seems to have little to do with the prevention of the larval infection.

TABLE 3.—*Passive transfer of immune rat serum absorbed or not absorbed with cysticerci.*

5 Group rats each 70–100 g	Rats were given on day before infection with ova of *T. taeniaeformis*:	Average cyst in each group 20 days after infection
1	0.8 ml immune serum #20 absorbed with living cysts	2
2	0.8 ml immune serum #20	1.3
3	0.8 ml immune serum #36 absorbed with living cysts	0
4	0.8 ml immune serum #36	0.3
5	0.8 ml lyophilized immune serum	0
6	0.8 ml normal serum	200
7	0.8 ml normal serum	210
8	No serum	259

A second experiment was then set up to ascertain whether the cysticercocidal substance takes an active part in destroying parasites in the liver. The therapeutic effect of specific immune serum on *C. fasciolaris* has already been studied by Miller (1932 and 1934), Miller and Gardiner (1934) and Campbell (1938c). Miller found that larvae failed to develop and were destroyed by immune serum administered 9 days after infection, but that they developed normally when immune serum was given 10 through 18 days after infection. Campbell confirmed this work. He further found that the therapeutic value of absorbed immune serum only differed from that of untreated immune serum in that fewer parasites were destroyed before encystment. In the present experiments, immune serum was not given until the 15th day because dead cysts like "spots" are fairly common in initially infected rats while larger dead parasites are less common. To attempt to offset the late start, multiple doses of serum were given. As may be seen in table 4, the rats in groups 3 to 5 were infected and 15 days later were given 5 increasing daily doses of untreated immune, lyophilized immune and normal serum, respectively, and groups 1 to 2 were treated similarly except that the serum was absorbed with living or treated cysts (table 4). The results are interesting although inconclusive because of the small number of animals. They indicate that untreated immune serum destroyed the largest number of cysts. Because the dead cysts in column 2 were unquestionably dead at the time of administering the first dose of serum, the effect of the serum is measured by

TABLE 4.—*Therapeutic effect of immune rat serum absorbed or not absorbed with cysticerci on infection with* T. taeniaeformis.

Group 4 rats each	Rats, weighing 90–125 g 15 days after infection, were given 5 daily intraperitoneal injections of 0.5, 1, 1.5, 2 and 3 ml	Serum absorbed with	Cysts found at autopsy 3 weeks after infection and 2 days after the last injection of serum					
			1 Living cysts	2 Dead cysts, pin-head in size—"spots"	3 Dead cysts, white and opaque	4 Cysts* showing a white spot to one side of body	Total	Percent Columns 3 and 4 of total cysts
1	Pooled immune serum	Living cysts	124	14	5	31	206	15
2	Pooled immune serum	Lyophilized cysts and ground fresh cysts	82	8	27	45	162	44
3	Pooled immune serum		60	9	35	47	151	54
4	Lyophilized pooled immune serum		40	4	15	6	55	36
5	Normal rat serum		116	15	4	33	168	22

* These cysts were still living, but the whiteness on their scolex was taken as the first sign of their eventual degeneration.

the results in columns 3 and 4. Absorption with living cysticerci reduced the percentage of affected cysticerci to about the level found in rats receiving normal serum. Absorption with wet and lyophilized cysticerci gave questionable results. There is some evidence that lyophilizing the serum reduced the therapeutic activity of the serum in the same manner that it reduced in vitro cysticercocidal activity. Absorption of the therapeutic action of immune serum on live cysticerci with living cysts which also absorb the in vitro cysticercocidal property indicates that the anticysticercal property may play a role in late immunity, i.e., it may destroy the parasites after they have settled in the liver. The author regrets that circumstances prevented him from repeating these experiments until statistically valid results proving or disproving this hypothesis could be obtained.

DISCUSSION

The presence of a bladder in *C. fasciolaris* separates it from metazoan parasites which are known to react with immune serums, such as nematodes, trematodes and glochidia. Various degenerative changes of the cysticerci in immune serum involve first a collapse and contracting of the bladder which, in turn, are followed by extrusion of the bladder cells, possible precipitates and death. In view of the formation of precipitate during the cysticercocidal action of saline as well as immune serum, it is impossible to decide if immune precipitates are formed as would be expected from the results on other parasites. If it occurs, it is apparently unimportant and appears late. The sequence of events is in some respects similar to that of glochidia when subjected to the blood of certain fish from which they derive their nourishment (Reuling, 1919). Reuling noted that when glochidia are placed in immune blood, the entire destruction of the parasite is initiated by the slow desquamation of cells of the mantle layer and adductor muscle. It also has some similarities to the activity of immune serum on the cercariae of schistosomes (Papirmeister and Bang, 1948). It seems most likely that the cysticercocidal property in immune serums, as contrasted with that in normal serums, is an antibody. The most puzzling fact in its behavior is the variation after heat or age inactivation and after the addition of complement. The author regrets that he has been unable to analyze its behavior more fully.

The experiments in the present paper indicate that a cysticercocidal substance can be demonstrated in immune serums except from rats too lightly infected or too heavily infected with living larvae. As mentioned previously, the latter condition may represent an essentially nonimmune animal, a poor antibody-producer or neutralization of the antibody by antigen. The cysticercocidal substance is heat stable at 56 C, although its potency may be somewhat reduced, and is susceptible to the lyophilizing process. Complement, on the other hand, is heat labile and withstands lyophilizing to a large extent. Immune serum when lyophilized retains its precipitin titer and protective property in vivo. In addition, immune serums which persistently give a negative reaction in vitro protect rats from becoming infected. These differences tend to show that the protective antibody is probably not associated with the cysticercocidal substance. This conclusion is further supported by experiments involving artificial immunization. It will be recalled that cysts remain normal when placed in serum from artificially immunized rats. Campbell believes that differences between the anti-

bodies of infected and artificially immunized animals depend chiefly upon the physiological condition of the inciting agent and that antigenic substances are produced by the growing organism over a period of several weeks until sufficient amounts are elaborated to induce antibody formation. Thus, he distinguished two kinds of immunity, i.e., early and late, in infected animals and only the early one in artificially immunized rats (Campbell, 1936).

The cysticercocidal substance may be associated with Campbell's (1938a) late immunity. However, it was regularly detected in vitro on the 7th day and, in one instance, as early as the 4th day, whereas late immunity was detected in vivo by Campbell (1938a) only after several weeks of infection as evidenced by the greater destruction of parasites in rats receiving 28-day serum than in those receiving 11-day serum. This seeming discrepancy may be due to the different methods used. Thus, it is possible that the cysticercocidal effect may be demonstrated in vitro earlier than in vivo. To test this conclusion, the therapeutic experiment was carried on in the last section and is suggestive though inconclusive.

CONCLUSIONS

A specific cysticercocidal property was generally found in immune serum from rats moderately infected (not too lightly or too heavily) with the larval form of *Taenia taeniaeformis*. It did not affect ova, but in in vitro hanging drop preparations, it collapsed and shrank the bladder of cysticerci and then in turn caused the extrusion of the bladder cells and death of the cysticerci. It was demonstrated 7 days after infection and as early as 4 days in one instance. It was less effective against old cysticerci. It largely withstood heating at 56 C for one hour but not lyophilization. It usually acted more rapidly in inactivated serums to which complement was added in a final dilution of 1:30. It was present in serums from rats infected 6 months previously or in serums kept in the refrigerator 5½ months.

A somewhat similar property was found in fresh normal rat and guinea pig serum and in saline. The serum property was not stable at 56 C, was not restored by complement in dilutions of 1:30, and disappeared when serums were refrigerated a week or more.

The specific cysticercocidal property did not account for the protective or precipitin properties of immune serum but may be associated with the late immunity against *Cysticercus fasciolaris*, which was differentiated by Campbell (1938b and c).

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STOOL VIRUS RECOVERY IN SUBCLINICAL POLIOMYELITIS DURING INCUBATION, FEBRILE, AND CONVALESCENT PERIODS

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Poliomyelitis virus has been reported to be present in the stools of a high percentage of paralytic and "abortive" (meningitic) cases during the acute phase of the disease and less frequently one week and more after the onset of symptoms,[1] although it has been shown to persist in the stools of some individuals for several weeks to months after the acute attack.[1–4] Virus has also been isolated from the stools of patients with the paralytic disease taken for periods up to 19 days before the onset of paralysis.[5,6]

Much less is known about the duration of excretion of virus in the stools of patients with subclinical poliomyelitis, although there are numerous reports of the isolation of virus from the stools of apparently healthy individuals and from "abortive" (meningitic) and subclinical cases known to have had none or varying amounts of contact with clinically recognized cases of the disease.[7] Virus has been isolated from the stools of subclinical cases for periods up to 19 days before onset of the disease[8,9] and one to two months after an attack[10] and from a healthy individual exposed to a clinical case one and two months before stools were collected.[11]

The present report describes the testing for virus of stools from 83 child contacts[12] of recognized clinical cases of poliomyelitis. The contacts tested were chosen because each developed one or more signs or symptoms compatible with subclinical poliomyelitis[13] within 3 weeks after exposure.

MATERIALS AND METHODS

The details of the epidemiologic study have been described elsewhere.[13] Briefly, it consisted of daily visits by a physician or a nurse to contacts of poliomyelitis cases, at which time clinical observations were made and specimens collected for virus study.

The method of storage of the specimens and the techniques used to prepare them for animal inoculation have been published.[14]

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Pooled stools of 83 child contacts who developed symptoms compatible with subclinical poliomyelitis were tested for virus by monkey inoculation. Two monkeys, either two rhesus (*Macaca mulatta*) or one rhesus and one cynomolgus (*Macaca cynomolgus*), were used to test the stools from each child. The monkeys were inoculated, observed in the same manner as described previously, and sacrificed after 21 days.[14]

Stools from a child were said to have contained virus if, following inoculation, one or both of the monkeys developed paralysis and had characteristic microscopic lesions or if characteristic lesions were seen in the absence of previous paralysis. The latter occurred in 8 of 154 monkeys examined microscopically, and in 4 cases the conclusion that the specimen contained virus rested on this finding alone. These lesions consisted of neurocytophagia of ganglion cells, perivascular collars and focal necroses of monocytes and neutrophiles in the medulla and in the gray matter of one or more levels of the spinal cord.

RESULTS

Of the 83 child contacts who developed symptoms of subclinical poliomyelitis, virus was recovered from the stools 4 of 10 (40%) collected during the week prior to onset of the first symptom or fever, from 20 of 24 (83%) collected on the day of onset, from 14 of 26 (54%) collected during the first week after onset, from 6 of 9 (67%) collected during the second week after onset, from 5 of 9 (55%) collected during the third week after onset, and from 1 of 3 (33%) collected during the fourth week after onset (table 1).

DISCUSSION

All of the 83 children whose stools were tested for the presence of virus were contacts of reported clinical (paralytic or meningitic) cases of poliomyelitis. All developed symptoms compatible with subclinical poliomyelitis.[13] One had transient paralysis and 2 had meningitic signs. Of the 83 children, 80 were not reportable clinical cases, and it is possible that the other 3 would not have been recognized and reported had they not been a part of the field study.

A very high percentage of the subclinical cases in this study were excreting virus in their stools at the time of onset of the first symptom or fever (table 1). This percentage diminishes sharply during the first week after appearance of the first symptom or fever in a manner very similar to that reported for the clinical disease.[1]

TABLE 1.—*Poliomyelitis virus in the stools of patients with the subclinical disease during the incubation, febrile, and convalescent periods.*

Period of collection	Number tested	Virus recovered	Percent with virus in stools
2–3 weeks prior to onset	2	2	
One week prior to onset	10	4	40
Day of onset	24	20	83
First week after onset	26	14	54
Second week after onset	9	6	67
Third week after onset	9	5	55
Fourth week after onset	3	1	33
Total	83	52	

In figure 1, the virus excretion rates in subclinical poliomyelitis reported here and from clinical cases reported by Horstmann et al[1] have been charted graphically and a theoretical curve drawn freehand. Virus excretion in the stools of clinical and subclinical cases appears to be similar.

In figure 2, the periods of collection of stools from the subclinical cases reported have been charted in relation to the observed onset of the first symptom or fever. A pool of stools from any child tested was listed in table 1 as tested at onset if it contained a stool taken at onset. Those pools containing no stool taken at onset were reported in table 1 in the period closest to onset in which a stool was taken, irrespective of the number of daily stool specimens contained in the pool.

If the stool pools listed on figure 2, which contain a stool taken on the day before the onset of the first symptom or fever, are examined, it will be seen that 15 of the 16 (94%) tested contained virus, indicating that the highest excretion rate of virus in the stools of sub-

clinical cases is just prior to onset of the first symptom or fever. The theoretical curve in figure 1 was drawn to indicate this probable peak excretion rate of stool virus just before onset.

The high percentage (83%) of isolations of virus from the stools of subclinical cases on the day of onset of the first symptom or fever indicates that the technical methods used are at least 83% effective, and that the amount of virus being excreted by subclinical cases at the time of onset of symptoms is probably as much as that being excreted at the onset of symptoms in clinical cases. This would indicate that virus multiplication in subclinical cases, at least in certain undetermined sites, is as great as in the clinical disease and suggests that immunity resulting from such a subclinical attack of the disease could be as good as that following the clinical disease.

Immunity acquired by a subclinical attack of the disease was early suggested as explaining the observed epidemiologic pattern of the disease. Wickman[15] recognized the subclinical cases during epidemics, especially among sibling contacts of paralytic cases, and pointed out that it was impossible to account for the epidemiologic behavior of poliomyelitis unless the latent and atypical infections were considered. The numerous reports of the presence of virus neutralizing antibodies in the serums of

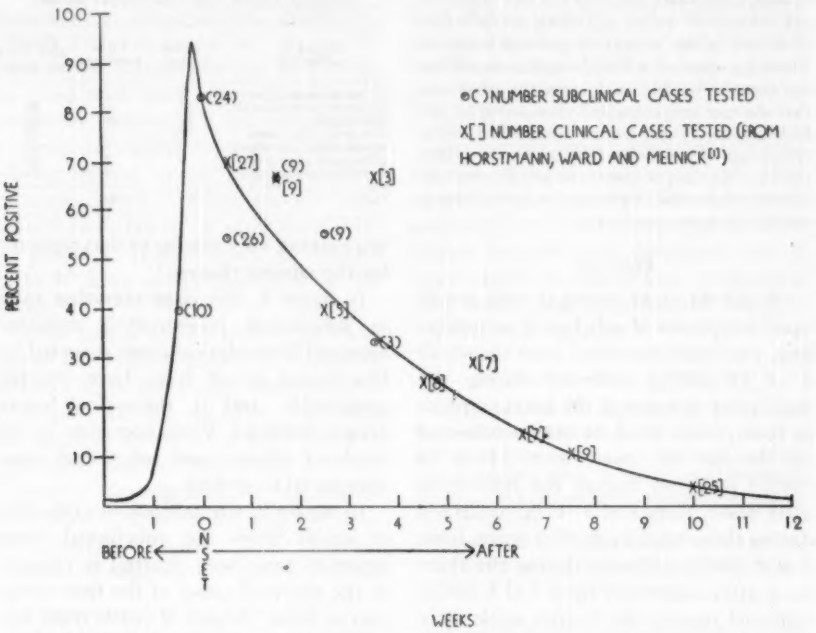

FIG. 1.—Isolation of the virus of poliomyelitis from the stools of patients during the incubation, febrile, and convalescent periods (61 hospitalized paralytic and meningitic cases and 83 subclinical cases).

15. Wickman, I. 1907, Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, Karger.

a high percentage of adults from all over the world[16–18] without history of an attack of poliomyelitis have been presented as direct evidence of the widespread occurrence of unrecognized infections resulting in immunity. The studies of Stocks in Britain[19] and Collins in America[20] point out that there are probably over 100 subclinical cases of poliomyelitis in endemic areas for every paralytic case. Data presented here indicate a stool virus excretion rate in subclinical cases very similar to that observed in clinical cases and offer further evidence that subclinical attacks of the disease can account for the observed widespread immunity in adults. Virus recovery from stools in a sampling study

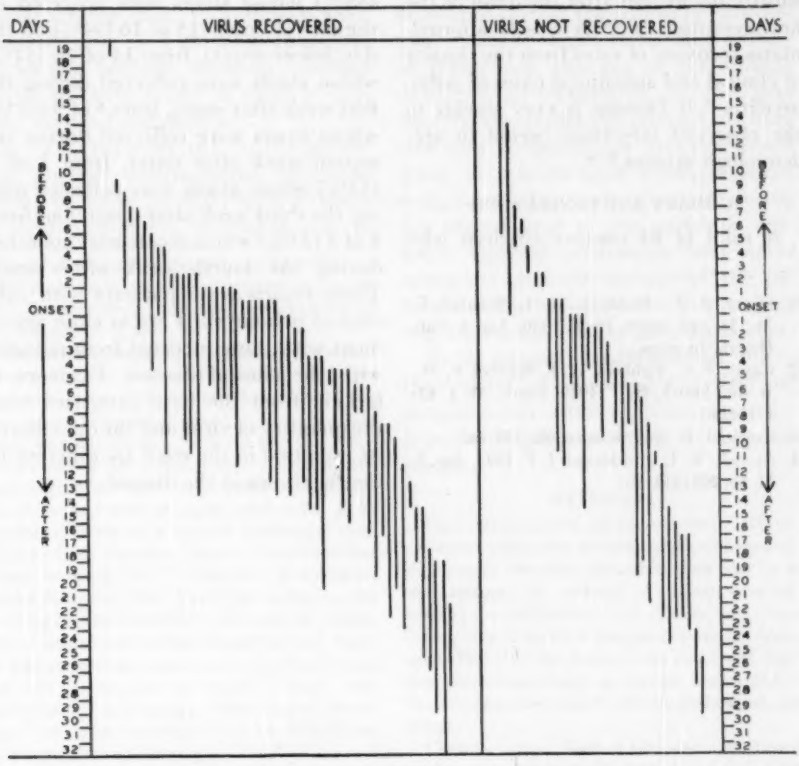

FIG. 2.—Stool collection intervals on 83 contact children (½ to 13 years of age) in relation to the onset of the first of one or more signs or symptoms compatible with subclinical poliomyelitis within 3 weeks after exposure.

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of the child population of Chicago during 1945 and 1946 offers further data to show that subclinical infections are very numerous in endemic areas.[21]

The high percentage of virus recovery from the stools of subclinical cases shortly before and after the onset of the first symptom or fever closely approximates recovery of virus from the throats of clinical and subclinical cases of poliomyelitis.[22] It likewise is very similar to the observed infectious period in epidemiologic studies.[23,24]

SUMMARY AND CONCLUSIONS

A total of 83 contact children who developed one or more symptoms of subclinical poliomyelitis were tested for stool virus. Virus was recovered from 4 of 10 (40%) whose stools were collected during the first week prior to onset of the first symptom or fever, from 20 of 24 (83%) whose stools were collected on the day of onset (15 of 16 [94%] on the day before onset), from 14 of 26 (54%) whose stools were collected during the first week after onset, from 6 of 9 (67%) whose stools were collected during the second week after onset, from 5 of 9 (55%) whose stools were collected during the third week after onset, and from 1 of 3 (33%) whose stools were collected during the fourth week after onset. These results from patients with subclinical poliomyelitis are in close agreement with those reported from patients with the clinical disease. Evidence is lacking from the data presented that the quantity of virus and the duration of its excretion in the stool are different in the two types of the disease.

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OCCURRENCE OF COLD AND STREPTOCOCCUS MG AGGLUTININS IN INFANTS WITH GASTROENTERITIS

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An outbreak of gastroenteritis which lasted two years was observed in an army camp.† During the first year several infants died; 11 autopsies were obtained, and in 8 of these cases the pulmonary sections revealed changes similar to those found in cases of primary atypical pneumonia.

In 1943, Peterson and his coworkers[1] and Turner[2] demonstrated that the serums of many patients with primary atypical pneumonia showed a rise in cold agglutinins, and in the same year Thomas and his associates[3] found that agglutinins for streptococcus MG also developed during the course of this disease. Because of the unexpected finding of pulmonary changes in the infants dying of gastroenteritis in the first year, it was thought of interest to study cold and streptococcus MG agglutinin titers in infants with gastrointestinal symptoms who were observed during the second year of the outbreak. Primary atypical pneumonia was never suspected clinically in any of the patients studied. No deaths due to gastroniteritis occurred in the second year.

As far as could be determined, this is the first report concerned with cold and streptococcus MG agglutinins in infants with gastroenteritis of unknown etiology.

MATERIALS

The infants studied, all dependents of military personnel, contracted gastroenteritis while traveling through the army station on their way to or from Europe. The majority of patients were billeted at the station from 3 to 12 days, and it was during this time that the gastroenteritis developed. Many of the infants who developed diarrhea were hospitalized to prevent the spread of the infection even though the symptoms were not severe.

Clinical course.—Most of the infants developed evidence of gastroenteritis from 1 to 6 days after arrival at the station. Both adults and infants were afflicted. In the former, vomiting and diarrhea, which were the chief symptoms, usually subsided by the third day. The infants, however, continued to have these symptoms for weeks and occasionally months. Some of the infants required frequent parenteral administration of fluids. The diarrhea was manifested by 4 to 20 liquid or loose

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TABLE 1.—*Cases exhibiting elevated streptococcus MG agglutinins.*

Case	Age in months	Sex	Days in hospital	Severity of diarrhea	Cough	X rays		Complications				Titer of streptococcus MG and cold agglutinins								
						Number taken	Findings	Pharyngitis	Otitis	Bronchitis	Other	Week of illness 1	2	3	4	5	6	7	8	9
1	8	M	37	Mild	Slight	1	Normal	2+	1+		1° vaccination			64, (64)		32				
2	9	F	70	Severe	Slight	2	Normal		2+		1° vaccination			128		0, (128)		0*		
3	3	M	63	Moderate	None	1	Normal		1+					0		256		32		0
4	9	M	43	Moderate	Slight	1	Normal	1+	1+					256		256		0		
5	12	F	25	Mild	None	2	Normal	1+	1+				128							
6	8	M	45	Moderate	Moderate	3	Normal	2+	1+	2+				0		32				
7	14	F	14	Mild	None	0							16							
8	10	M	20	Mild	None	1	Normal	1+	2+				16		0					
9	11	M	44	Moderate	None	1	Normal	1+	2+				256		0, (64)			0		
10	10	M	12	Moderate	Little	0		2+		1+			64, (256)	16						
11	3	M	23	Mild	Slight	1	Normal	2+	4+				64							
12	12	M	46	Moderate	None	3	Normal		1+							640, (80)	80			
13	8	M	46	Moderate	None	0		1+	1+					320			0			
14	6	M	49	Severe	Severe	2	Normal	2+		1+	Pyelitis						640			
15	12	F	57	Moderate	Moderate	2	Normal	2+	2+					80		320				
16	8	M	89	Severe	None	4	Normal						0			64		64		
17	12	M	43	Moderate	Moderate	3	Normal	1+	1+				64							
18	9	M	35	Severe	Severe	2	Normal	1+	1+	4+							1280		0	0
19	7	M	54	Severe	None	0		2+				0			64		0			
20	6	F	72	Severe	Slight	2	Normal	1+	1+		Pyelitis				256		64			0
21	10	M	55	Mild	None	1	Normal	1+	1+			0	16		0	0				
22	12	F	32	Mild	None	1	Normal	1+	1+					80						
23	6	M	16	Mild	Moderate	1	Normal		2+			0	0	16						
24	12	M	18	Mild	None	4	Normal		1+	2+					64, (256)					

1+ to 4+ indicates varying degrees of severity.
Figures in parentheses represent cold agglutinin titers.
* 0 is streptococcus MG titers less than 1:10.

TABLE 2.—*Cases exhibiting streptococcus MG agglutinins in a titer of less than 1:10.*

Case	Age in months	Sex	Days in hospital	Severity of diarrhea	Cough	X rays		Complications				Titer of streptococcus MG and cold agglutinins									
						Number taken	Findings	Pharyngitis	Otitis	Bronchitis	Other	Week of illness 1	2	3	4	5	6	7	8	9	10
25	6	M	19	Mild	Moderate	1	Normal		1+				0	0		0					
26	6	M	43	Moderate	None	1	Normal	1+				0		0	0		0				
27	8	M	50	Moderate	None	1	Normal	1+					0		0		0				
28	6	M	44	Moderate	None	0		1+	1+			0		0		0					
29	8	M	80	Severe	Moderate	2	Normal	2+	2+	4+	Pyelitis		0			0		0		0	
30	7	M	37	Moderate	Slight	1	Normal	2+	2+		Asthma	0		0	0	0					
31	24	M	55	Moderate	None	1	Normal	1+	1+			0		0							
32	2	F	39	Mild	None	2	Normal		1+					0	0		0	0			
33	8	F	25	Mild	Moderate	2	Normal	1+	1+				0								
34	8	F	52	Mild	Slight	2	Increased markings	2+		4+					0						
35	8	M	14	Moderate	None	0		2+	2+		1° vaccination			0							
36	10	M	15	Moderate	Moderate	1	Increased markings			3+	Asthma	0	0								
37	7	M	43	Moderate	Slight	1	Normal	2+					0	0							
38	9	F	40	Mild	None	1	Normal	2+	1+				0					0		0	
39	11	M	16	Mild	None	0			1+			0									
40	6	M	50	Severe	Moderate	3	Normal	2+		4+							0				
41	3	F	46	Severe	Slight	1	Normal	1+	1+			0									
42	6	F	20	Mild	Slight	2	Normal	1+	1+	2+			0								
43	12	M	14	Mild	None	0						0									
44	12	M	18	Mild	None	4	Normal		1+	1+					0, (256)						

See table 1 footnotes.

yellow, green or clear-colored, nonbloody, nonmucoid stools per day. Signs of a mild upper respiratory infection developed in many of the infants after the onset of the gastroenteritis. Stomatitis was not observed. The mild cases were usually afebrile, but the severe cases, especially those who were markedly dehydrated on admission, had periods of pyrexia. The clinical response to penicillin in these cases was not striking, but sulfadiazine often seemed to reduce the length of the febrile period but only infrequently the severity of the diarrhea.

Laboratory findings.—The white blood counts in most cases were normal, but a few cases showed a leucopenia. Urinalyses were normal. Throat cultures usually failed to reveal pathogenic bacteria although *Escherichia coli* was found in many cases who were vomiting. Erythrocyte sedimentation rates usually were not elevated. Chest X rays were taken routinely and repeated when respiratory symptoms or signs developed. In most cases no abnormalities were disclosed. A few cases showed increased bronchovascular markings. Blood cultures and agglutinations for enteric pathogens were negative. Frequent stool cultures as well as examinations for ova and parasites failed to reveal any intestinal pathogens. Nasal washings from 7 infants were pooled and inoculated into monkeys, guinea pigs, rabbits, hamsters and mice by intranasal, subcutaneous, intraperitoneal, intracerebral and corneal routes and into embryonated eggs by the amniotic, allantoic and chorioallantoic routes. No evidence of an infectious agent was obtained by any of these procedures.* Since no human volunteers were inoculated, it was impossible to exclude the presence of the virus of primary atypical pneumonia.[4]

METHODS

Streptococcus MG agglutinins.—Agglutinins against streptococcus MG were determined as described by Thomas and his associates.[5] Titers over 1:10 dilution were considered significant. No attempt was made to isolate streptococcus MG from any of the patients studied.

Cold agglutinins.—Cold agglutinins were determined by the method described by Peterson et al.[1] Titers over 1:60 were considered significant.

RESULTS

Table 1 summarizes the important clinical findings and the streptococcus MG agglutinin titers on the patients who developed a titer of 1:16 or more.

Streptococcus MG agglutinins were not present during the first week of illness but rose sharply and became positive in 80% during the second week. They reached a maximum in the third week and thereafter declined slowly. In general the height of the streptococcus MG agglutinin titer paralleled the severity of the gastroenteritis but was not correlated with the presence of bronchitis.

Table 2 lists the data in those patients in whom no streptococcus MG agglutinins were demonstrated at any time during the illness but who showed a clinical course indistinguishable from those depicted in table 1. Table 2 shows that in 17 out of the 20 patients the serums were studied during the 2nd to the 7th weeks, the time when the infants shown in table 1 exhibited the maximum titers. In 3 of these 17 patients the gastroenteritis was severe, and in 6 others in this group the disease was complicated by bronchitis. In 3, the absence of streptococcus MG agglutinins may have been due to the fact that the serums were obtained during the first week of the disease.

Cold agglutinins.—Cold agglutinins were obtained in only 7 patients because the blood in the remaining tests had been refrigerated before the serums were separated. All 7 of the serums, obtained between the 2nd and 5th week of illness, which were properly handled (see cases 1, 2, 9, 10, and 24 in table 1 and case 44 in table 2) showed cold agglutinins in a dilution of 1:64 to 1:256. Six of these 7 patients showed positive streptococcus MG agglutinins

* These studies were carried out under the direction of Dr. J. E. Smadel, Director of Virus and Rickettsial Laboratory, Army Graduate School.

4. Commission on acute respiratory disease: The transmission of primary atypical pneumonia to human volunteers, 1945, Bull. Johns Hopk. Hosp. **79**: 153–167.
5. Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E. and Horsfall, F. L., Jr. 1945, Studies on primary atypical pneumonia. II. Observations concerning the relationship of a non-hemolytic streptococcus to the disease. J. Clin. Investigation, **24**: 227.

at some time during the course of their illness, but in 2 instances the cold agglutinins were present after the streptococcus MG agglutinins had disappeared (cases 2 and 9, table 1). In the 7th patient (see case 44, table 2) with positive cold agglutinins but with negative streptococcus MG agglutinins, only one serum was available for the latter determination.

DISCUSSION

These patients were considered to have had an infectious diarrhea. The failure to culture bacterial pathogens from the stools, to demonstrate agglutinins against salmonella, the afebrile course and the absence of blood and mucus from the stools argues strongly against salmonella or shigella enteritis. The absence at the onset of gastroenteritis of a respiratory infection ruled out a parenteral infection. The epidemiology, normal and occasionally depressed, white blood cell count, afebrile course and lack of response to penicillin suggested a virus etiology in spite of the fact that no virus was demonstrated by egg or animal inoculation. The consistently negative X ray findings and the absence of cough made it seem unlikely that the agent causing diarrhea in these patients was identical with that of primary atypical pneumonia. The serological reactions encountered in this group of patients, however, were similar to those seen in cases of primary atypical pneumonia. Horsfall[6] in his review showed that 48% of patients with primary atypical pneumonia developed agglutinins against streptococcus MG, and Morgan and Finland[7] in a more recent review showed that 80% of patients with primary atypical pneumonia developed streptococcus MG agglutinins. Fifty-four percent of the patients in the present study had agglutinins against the MG streptococcus.

Too few cold agglutinin determinations were available to evaluate their significance. In view of the studies of Thomas and his coworkers it seems unlikely that the serological changes were caused by anamnestic reaction.[3]

Evidence in favor of a virus etiology as a cause of certain cases of gastroenteritis has been reported from several laboratories.[8–10] Although the finding of elevated cold and streptococcus MG agglutinins in the patients described in this report does not warrant the conclusion that a specific virus is necessarily the etiological agent, it suggests that such serological studies may prove useful in differentiating a special group of patients with gastroenteritis.

SUMMARY

1. Elevated cold and streptococcus MG agglutinins were found in infants with gastroenteritis of unknown etiology.

2. The significance of elevated cold and streptococcus MG agglutinins in this disease was not determined, but they appeared to offer a new approach to the problem of gastroenteritis of unknown etiology.

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ATTEMPTS TO PRODUCE LYMPHOCYTOPENIA IN RABBITS FOLLOWING THE INTRAVENOUS INOCULATION OF CERTAIN VIRUSES

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The occurrence of lymphocytopenia in rabbits following the intravenous inoculation of influenza virus and the prevention of this response with type-specific immune serum has recently been reported by Harris and Henle.[1] Similar lymphocytopenic responses were also demonstrated after the injection of mumps virus, rough pneumococci, and nucleoproteins derived from hemolytic streptococci.†

The possibility existed that this phenomenon might be evoked by a wide number of infectious agents. Of particular interest to us was the consideration that it might be useful in the study of the causative agents of poliomyelitis, infectious hepatitis, and infectious mononucleosis. The purpose of this paper is to report the results of experiments with materials suspected of containing the etiological agents of these diseases as well as with Newcastle disease, vaccinia, and influenza viruses. In addition, mention is made of results with Coxsackie virus (C virus) isolated from stools of patients with aseptic meningitis.[3]

MATERIALS AND METHODS

White male rabbits weighing from 3 to 5 pounds were employed. The material to be tested was inoculated into a marginal ear vein, and blood samples were obtained from the marginal vein of the opposite ear before inoculation and usually at intervals of 4, 6, and 24 hours afterwards. Duplicate white counts were made on each blood sample, and 200 leucocytes were counted on smears stained with Giemsa.

With influenza virus as a model,[1] a response to an agent tested was considered significant if a drop of over 60% in the absolute number of lymphocytes occurred within 6 hours after inoculation and returned toward normal by the 24th hour.

The agents used in this study, their source, and their infectivity titer (ID_{50}) are listed below:

1. *Influenza virus* (PR8 Strain): allantoic fluid, infectivity titer for eggs $10^{-8.0}$, chicken cell hemagglutination titer 1:1024.

2. *Vaccinia virus:* (a) rabbit testicle, 20% suspension, skin infectivity titer (MID) $10^{-7.7}$; (b) elementary bodies purified from rabbit skin by the method of Parker and Rivers,[4] rabbit skin infectivity titer 10^{-8}.

3. *Newcastle disease virus:* allantoic fluid, egg infectivity titer 10^{-8}, chicken cell hemagglutination titer 1:512.

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* National Institutes of Health Postdoctorate Research Fellow, U. S. Public Health Service.

1. Harris, S. and Henle, W. 1948, J. Immunol. **59:** 9–20.

† Since the completion of this paper, Wagner et al[2] have shown that the lymphocytopenia produced in rabbits by the injection of influenza virus is accompanied by fever and that Newcastle disease virus produces a similar febrile response.

2. Wagner, R. R., Bennett, I. L., Jr. and LeQuire, V. S. 1949, J. Exper. Med. **90:** 321–334.

3. Melnick, J. L., Shaw, E. W. and Curnen, E. C. 1949, Proc. Soc. Exper. Biol. & Med. **71:** 344–349.

4. Parker, R. F. and Rivers, T. M. 1935, J. Exper. Med. **62:** 65–72.

4. *Poliomyelitis virus:* (a) Lansing strain, mouse cords, concentrated 100 times by ultracentrifugation,[5] infectivity titer in mice 10^{-5}; (b) W. P. strain isolated in New York City, 1944, ultracentrifuged concentrate of monkey spinal cords; (c) NC, 1948 strain, ultracentrifuged sediments of human stools from poliomyelitis patients, titer in monkey 10^{-4}.

5. *C virus:*[2] (a) Botwinic strain (New Haven, 1948) mouse brain, titer in mice 10^{-4}; (b) Texas fly strain (1948), mouse muscle, titer in mice 10^{-4}.

6. *Hepatitis virus:* pooled, acute phase human serums. This material was kindly provided by Dr. Horace T. Gardner.

7. *Infectious mononucleosis:* pooled, acute phase human serums and chick embryonic fluids from the second egg (fluid) passage of pooled throat washings taken at the same time.

Controls: suspensions of normal monkey and ultracentrifuged mouse cords, normal human serum, desoxyribonucleoprotein obtained from normal spinal cord, ultracentrifuged samples of normal human stools and suspensions of *Bacterium coli* and *B. proteus*.

RESULTS

The results are indicated in table 1, in which it will be observed that the only viruses tested which produced marked lymphocytopenia were influenza and Newcastle disease viruses. The average drop in the absolute number of lymphocytes in 4 rabbits inoculated with 1.0 ml of allantoic fluid containing influenza virus was 80%, and the average drop in 11 rabbits following injection of a similar amount of allantoic fluid containing Newcastle disease virus was 70%. Both preparations had infectivity titers for eggs of 10^{-8} and agglutinated chicken red blood cells in dilutions of 1:512 to 1:1024.

TABLE 1.—*Tests for reduction in lymphocyte counts 4 to 6 hours following the intravenous injection of certain agents into rabbits.*

Agent*	Amount in ml	Number of rabbits	
		Inoculated	Lymphocytopenia†
Influenza virus	0.1–1.0	6	5
Newcastle disease virus	1.0–5.0	13	11
Vaccinia virus	0.1–5.0	4	0
C virus	1.0	10	0
Hepatitis	1.0	10	0
Infectious mononucleosis	0.5–5.0	10	0

* See text for preparation and source.
† Lymphocytopenia: number of rabbits showing 60% or greater drop in the absolute number of lymphocytes.

Lymphocytopenia of marked degree was also produced by ultracentrifuged preparations of stools from patients with poliomyelitis, but equally great responses were elicited by similar preparations of sterile normal human stools, as well as with suspensions of bacteria (*B. proteus* and *B. coli*), which are common contaminants of such material. There was no significant response to other control preparations.

DISCUSSION

The lymphocytopenia occasioned by the intravenous injection of influenza virus into rabbits as previously reported by Harris and Henle[1] has been confirmed. In addition, similar responses have been found after the inoculation of 1.0 ml or more of allantoic fluid containing Newcastle disease virus as well as with suspensions of *B. coli* and *B. proteus* and with ultracentrifuged suspensions of human stools from normal persons or from patients with poliomyelitis. Neither marked nor consistent lymphocytopenia was produced by the inoculation of preparations of poliomyelitis, vaccinia, or C viruses or with materials obtained from patients in the early, acute phase of viral hepatitis and infectious mononucleosis. These results suggest that this phenomenon can not be used as a nonspecific screening test for the presence of infectious agents in human material. It is perhaps significant that the viruses so far found capable of producing this reaction all possess a typical hemagglutinin.

5. Melnick, J. L. 1943, J. Exper. Med. **77**: 195–204.

CONCLUSIONS

1. Lymphocytopenia has been produced in rabbits following the intravenous inoculation of influenza and Newcastle disease viruses, suspensions of *Bacterium coli* and *B. proteus*, and ultracentrifuged preparations of human stools.

2. Lymphocytopenia of similar degree has not been observed following the intravenous injection of poliomyelitis, vaccinia, and Coxsackie (C) viruses, nor with material from patients in the acute phase of viral hepatitis or infectious mononucleosis.

THE TOXINS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP OF AGENTS.

I. THE TOXICITY OF VARIOUS MEMBERS OF THE PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP

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In 1943 Rake and Jones demonstrated a toxic factor in yolk sacs heavily infected with the virus of lymphogranuloma venereum. As mice are not infected by intravenous injection of this agent, the test mice either died soon after injection or recovered and showed no evidence of infection. In subsequent studies similar toxins were demonstrated in the yolk sac suspension of the viruses of mouse pneumonitis, meningopneumonitis and feline pneumonitis (Rake and Jones, 1944; Hamre and Rake, 1944). The toxic component is labile, being destroyed in a short time at incubator temperatures and by low concentration of formalin, and is present in high concentration only in the yolk sac. Each of the three pneumonitis viruses produced a characteristic graphic death curve when injected intravenously into mice, showing an initial interval in which deaths were due to toxin, a second interval during which few or no deaths occurred and a third interval during which the remaining mice died as a result of infection.

The toxins of each of the four viruses were antigenic, and toxin-neutralizing antiserums were prepared by the injection of toxic or formol-treated virus suspensions into rabbits and chickens. Rake and associates were unable to demonstrate neutralization of the four toxins by heterologous antiserums, whereas homologous toxin and antitoxin mixtures showed complete neutralization. Chorioallantoic membranes and allantoic fluid heavily infected with the feline pneumonitis virus are also toxic for mice (Hamre, Rake and Rake, 1947). This virus was adapted to the allantoic cavity by serial passage.

The demonstration of specific antigenic toxins associated with these viruses led to the studies on other members of the group reported here. Since a large number of the psittacosis-lymphogranuloma viruses are available at this laboratory, their toxicity for mice was studied.

MATERIALS AND METHODS

Virus strains.—Thirty-nine virus strains of the psittacosis-LGV group were tested for toxicity for mice. The history of each strain is given in the appendix.

Preparation of virus suspensions.—Yolk sacs were used for the preparation of all virus suspensions. Six- to 8-day embryonated hens' eggs were inoculated with 0.5 ml of a virus suspension in the dilution calculated to produce death in 3 or 4 days after inoculation when possible. All eggs dying within the first 48 hours thereafter were discarded, and beginning on the third day, all eggs were candled at frequent intervals. After death of half of the embryos, the yolk sacs of all eggs in the group were harvested and ground in a Waring Blendor for 1½ minutes as a 20% suspension in cold diluent. Aluminum Waring Blendor bowls fitted with a rubber-sealed screw cap were used for all grinding. A portion of the suspension

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was removed for staining and sterility tests, and the remainder was distributed in sterile acid-cleaned test tubes and stored at −70 C. Smears of all suspensions were made by the method of Macchiavello.

Selection of the diluent.—Rake and Jones (1943, 1944) reported that centrifuged yolk and egg fluids or serums from various sources could be used in the preparation of yolk sac suspension, and that the resulting suspensions were well tolerated by mice when injected intravenously.

Although the suitability of serum as a diluent had been confirmed by previous experiments, a simpler and more easily prepared medium was desired. The suitability of a 0.1 *M* sodium phosphate buffer at pH 6.8 containing 0.2% gelatin was determined, and 0.5 ml could be given to mice intravenously without serious effect. This was the diluent used for preparation of all virus suspensions employed in this study.

Mouse test for toxin.—White mice of one strain were used exclusively in these experiments. Serial lung passages had repeatedly showed the mice to be free from inapparent infections by viruses of this group. The mice weighed 12 to 18 g, and in each test mice of uniform size were selected.

Prior to testing for toxicity, the 20% yolk sac virus suspensions were thoroughly mixed and then centrifuged 3 to 5 minutes at 2000 r.p.m. in an angle-head centrifuge to remove all large particles. Serial twofold dilutions, beginning at a dilution of 1:10, were made in gelatin-phosphate buffer. The dilutions were calculated on the basis of weight of wet yolk sacs.

Prior to injection of 0.5 ml of the appropriate dilution, test mice were placed in an incubator at 37 C for approximately 15 minutes to allow dilation of the tail veins for intravenous injection. A small percentage of test mice died from shock or emboli immediately after injection. The remainder were active after a few minutes. Dead mice were removed at regular intervals and the numbers were recorded.

The toxicity of each virus suspension was calculated on the basis of the number of mice dying within 24 hours after injection; later deaths were attributed to infection.

RESULTS

The toxicity of at least 2 yolk-sac suspensions of most of the 39 virus strains listed in the appendix was determined.

Viruses isolated from man and other mammals.—The results of toxin tests with 22 strains from these sources are shown in table 1. For comparison, the highest dilution of each strain which caused death of half or more of the test mice within 24 hours after injection is given. Only 5 strains failed to kill mice in dilutions of 1:10 or greater. The 2 lymphogranuloma venereum strains showed a low infectivity for eggs when injected by the yolk sac route, and heavily infected suspensions were not obtained. Although the mouse pneumonitis (Nigg) culture was well adapted to yolk sac culture, it did not infect mice when given either intravenously or intraperitoneally.

TABLE 1.—*The toxicity of strains isolated from man and other mammals.*

Virus strain	Source	Number of suspensions tested	Number of mice	Maximal toxic* dilution (0.5 ml /mouse)
Louisiana	Human	17	442	1:80
S-F	Human	10	151	1:80
M.D.	Human	7	69	1:20
H.P.	Human	4	55	1:20
Illinois	Human	8	68	1:20
H-Se	Human	7	96	1:40
H-We	Human	7	97	1:40
H-Mu 46	Human	8	112	1:20
H-Jo	Human	6	63	1:20
H-Pa	Human	3	40	1:20
H-Br	Human	3	30	1:20
H-St	Human	11	140	1:40
H-Cl	Human	13	171	1:20
H-Ga	Human	2	36	<1:10
H-Ca	Human	2	36	<1:10
LGV-1	Human	5	47	<1:10
LGV-2	Human	1	9	<1:10
Meningopneumonitis	Human? Ferret?	10	112	1:20
Feline pneumonitis	Cat	9	101	1:40
Bar Harbor	Mouse	4	47	1:10
de Burgh	Mouse	1	8	1:20
Mouse pneumonitis (Nigg)	Mouse	2	18	<1:10

* Highest dilution killing half or more of the test mice within 24 hours after injection.

Viruses isolated from birds.—Five of the 17 strains failed to kill mice within 24 hours in dilutions of 1:10 or greater, and none of the strains were toxic in dilutions of 1:80 (table 2).

Graphic death curves produced by the intravenous injection of the various strains into mice

Rake and associates demonstrated graphic diphasic death curves of mice by plotting the percentage of total number of mice dying during intervals

after the intravenous injection of the mouse pneumonitis, meningopneumonitis and feline pneumonitis agents. A single phase curve was obtained with the lymphogranuloma venereum virus, as this agent apparently does not infect mice by the intravenous route.

TABLE 2.—*The toxicity of strains isolated from birds.*

Virus strain	Source	Number of suspensions tested	Number of mice	Maximal* toxic dilution (0.5 ml /mouse)
Pasadena parakeet	Parakeet	7	73	1:20
Washington parakeet	Parakeet	3	16	1:20
Seattle parakeet	Parakeet	6	46	<1:10
Schaefer parakeet	Parakeet	3	27	1:10
6 BC	Parakeet	2	35	<1:10
Detroit parakeet	Parakeet	8	104	1:10
P-Endo-1	Pigeon	8	90	1:40
P-Endo-3	Pigeon	12	170	1:40
P-Endo-4	Pigeon	1	9	1:20
P-Endo-D	Pigeon	1	10	<1:10
P-Au 46	Pigeon	3	29	1:40
P-207	Pigeon	4	59	1:20
Petaluma	Duck	3	21	1:20
Long Island	Duck	2	36	<1:10
Friend	Chicken	2	25	1:20
New York dove	Dove	1	24	<1:10
Washington canary	Canary	2	17	1:40

* Highest dilution killing half or more of the test mice within 24 hours after injection.

Similar graphic presentation of the results obtained with the various viruses in the present study also show some significant features. These curves are based on the total number of mice tested, and the number of mice dying during each interval is plotted as the percentage of the total. This method of presentation does not indicate the toxicities of various strains, but a definite diphasic curve apparently permits differentiation of deaths due to toxin and those due to infection.

Viruses showing diphasic death curves.—The results of toxin tests in mice with the Louisiana and Illinois human pneumonitis and the meningopneumonitis and feline pneumonitis viruses show diphasic death curves (fig. 1).

Over 50% of the 442 mice tested with the Louisiana strain died within the first 16 hours after injection; very few deaths occurred in the 17 to 24 hour period. By the end of 48 hours most of the remaining mice were dead or showed signs of illness, and the second death phase had begun. The latter deaths presumably resulted from infection.

Sixty-eight mice were used in tests with the Illinois strain. The curve differs from that obtained with the Louisiana virus in the delay of the second-phase deaths. The results with the feline pneumonitis strain show a diphasic curve similar to that obtained with the Louisiana virus. A diphasic death curve is also seen in the case of the meningopneumonitis virus, with the 2 peaks in the 0- to 24- and 72- to 96-hour periods.

The S-F type viruses.—Three viruses of the S-F type, the S-F, M.D. and H.P. strains (Meiklejohn et al, 1944), were tested in mice for toxicity. Results are presented graphically in figure 2. These strains are similar as

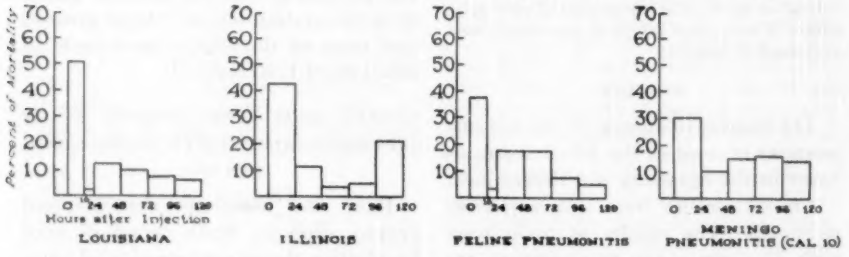

FIG. 1.—Mortality rate of mice during 24-hour intervals after injection of toxic yolk sac suspensions of the Louisiana, Illinois, feline pneumonitis and meningopneumonitis (Cal 10) viruses.

would be expected from the histories of the strains.

These results illustrate the rapidity with which toxins of this strain kill mice. The S-F virus consistently killed in a Waring Blendors, and suspensions from each were inoculated by both intracranial and intranasal routes into normal mice. These mice all remained healthy. Eaton and his associates (1941)

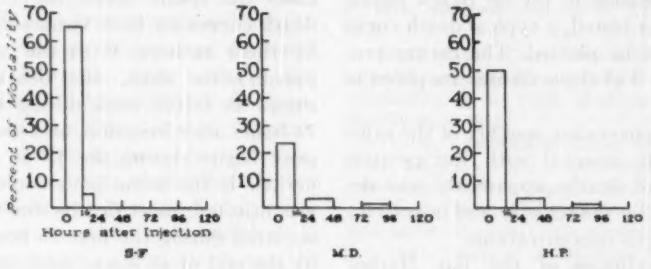

FIG. 2.—Mortality rates of mice during intervals after injection of toxic yolk sac suspensions of the S-F, M.D. and H.P. viruses.

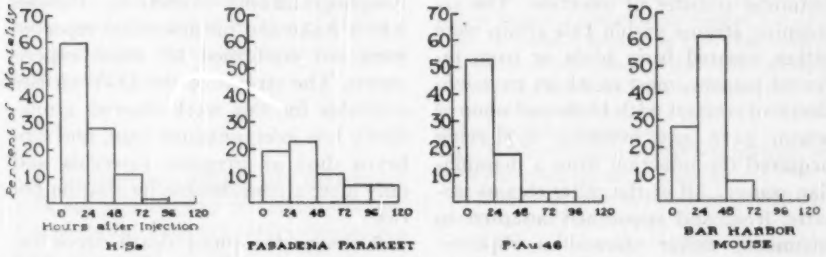

FIG. 3.—Mortality rates of mice during intervals after injection with toxic yolk sac suspensions of H-Se, Pasadena parakeet, P-Au 46 and Bar Harbor mouse viruses.

higher dilution than either of the other strains, but all killed mice within a short time in the lower dilutions. With all of these strains, few mice succumbed after 24 hours. Of those dying after 24 hours, none showed evidence of infection by these viruses. In the case of the S-F virus, evidence was obtained that survivors were not carriers of the virus 21 days after infection. Two groups of survivors of toxin tests were sacrificed 23 days after injection and examined for infection. All appeared normal. The spleens, livers, lungs and kidneys of 5 of these mice were pooled and ground in found mice to be insusceptible to intraperitoneal inoculation of the S-F virus, and showed that inoculation of the virus by this route does not produce a carrier state in mice. Wagner et al (1949) made similar observations after subcutaneous inoculation.

Other viruses which do not produce biphasic death curves.—This group apparently contains all of the other strains which were found to be toxic for mice. It includes strain isolated from patients who apparently had not become infected by man-to-man transmission (most of them gave a his-

tory of contact with various birds), 4 strains isolated from parakeets, 5 strains isolated from pigeons, 2 strains isolated from ducks, one strain isolated from chickens, one from a canary and the Bar Harbor mouse strain. As only one suspension of the de Burgh mouse strain was tested, a typical death curve could not be plotted. The curves produced by 4 of these viruses are given in figure 3.

These curves are roughly of the same type. The interval with the greatest number of deaths apparently was determined by the toxicity and infectivity of the virus concentrations.

The inclusion of the Bar Harbor mouse strain within this group is surprising as it is the only virus in the group involved in a definite mammal-to-mammal transfer of infection. The remaining strains within this group were either isolated from birds or from infected persons, most of whom gave evidence of contact with birds and none of whom gave any evidence of having acquired the infection from a mammalian source. All of the other strains isolated from and apparently adapted to mammals either showed a diphasic death curve or were not infectious for mice when injected intravenously.

DISCUSSION

Twenty-nine of the 39 strains tested killed mice consistently in less than 24 hours in dilution of 1:10 or greater, and these deaths cannot be reasonably attributed to infection. Neither can early deaths be attributed to the effects of the diluent or yolk sac tissue, as normal diluent and 20% suspensions of normal yolk sac have been injected into mice intravenously without serious effect. Also, on numerous occasions suspensions of ordinarily toxic virus strains have completely failed to cause early deaths, with little change in the time of occurrence of deaths due to infection.

These results confirm the observations of Rake and Jones (1944) and Hamre and Rake (1944) concerning the toxic effect of the meningopneumonitis and feline pneumonitis viruses. In both cases the results show the two-phase death curves for both viruses described by these authors. With the meningopneumonitis virus, the death curve shows an initial peak during the first 24 hours after injection, and the second peak begins during the 48- to 72-hour period. If the feline pneumonitis virus was injected, most deaths due to toxin occurred during the first 16 hours, and by the end of 48 hours, mice surviving the toxic effects began to die as a result of infection. The toxic effects of the mouse pneumonitis (Nigg) and the lymphogranuloma venereum viruses which Rake and his associates reported were not confirmed by these experiments. The strains of the LGV viruses available for this work showed a relatively low infectivity for eggs, and embryos died at irregular intervals and only after a considerable incubation period.

A similar two-phase death curve has been shown in these experiments for the Louisiana and Illinois viruses. With the Louisiana virus almost all toxic deaths occurred during the first 16 hours, and by the end of 48 hours the infective deaths occurred in considerable numbers. With the Illinois virus, the peak period for infective deaths came 96 to 120 hours after injection.

With the S-F, M.D. and H.P. viruses, practically all deaths occurred within the first 16 hours, and the survivors of the toxin tests were not found to be infected. The behavior of S-F group of viruses when injected intravenously into mice is of especial interest, and the failure to infect mice by this route may well represent extreme adaptation of

the virus to the tissue of the respiratory tract, with a consequent loss of infectivity in the reticulo-endothelial cells.

None of the other viruses tested showed definite diphasic death curves. In the case of some strains, the number of mice tested and the frequency of collecting dead animals were insufficient to detect a diphasic type curve. It is significant, however, that none of the strains isolated from birds or from patients who gave histories of bird contact showed this two-phase phenomenon. With the exception of the Bar Harbor mouse strain, however, all of the viruses which were apparently established in one mammalian species did show a diphasic death curve.

Of further interest is the fact that of the 15 psittacosis strains isolated from man, 13 were consistently toxic for mice, and the other 2, the H-Ga and H-Ca strains, were toxic to some extent in low dilution.

SUMMARY

A method for testing the toxicity of yolk sac suspensions of psittacosis and lymphogranuloma venereum viruses has been described. A total of 39 viruses from human, avian and mammalian sources were tested in mice by this technique. Characteristic two-phase death curves were found only for the Louisiana, Illinois, meningopneumonitis (Cal 10) and feline pneumonitis viruses.

All of the 15 strains isolated from man showed evidence of some toxicity for mice.

APPENDIX

Histories of the virus strains.—Thirty-nine viruses of this group have been tested for toxicity in mice and a brief description of each follows. Most of these were isolated at the Hooper Foundation by Dr. B. Eddie, and those not otherwise designated were obtained from this collection.

1. Louisiana.—Isolated by Olson and Larson (1945) in an outbreak of human pneumonitis. Received from Dr. D. J. Davis, National Institute of Health, in 1946.

2. S-F.—Isolated by Eaton et al (1941) in a case of human pneumonitis. Received from Dr. M. D. Eaton in 1940.

3. H. P.—Isolated from laboratory worker infected by contact with S-F strain (Meiklejohn et al, 1944). Received from Dr. G. Meiklejohn in 1948.

4. M. D.—Isolated from nurse infected by contact with a patient with pneumonitis of S-F type (Meiklejohn et al, 1944). Received from Dr. Meiklejohn in 1948.

5. Illinois.—Isolated by Zichis and Shaughnessy (1945) in a case of human pneumonitis. Received from Dr. H. J. Shaughnessy.

6. H-Jo.—Isolated in 1945 from patient who gave history of contact with pigeons.

7. H-Pa.—Isolated in 1945 from patient who gave a history of contact with pigeons and chickens.

8. H-Br.—Isolated in 1945 from a patient who gave a history of contact with pigeon.

9. H-St.—Isolated in 1946 from patient who gave a history of contact with pigeons.

10. H-Mu 46.—Isolated in 1946 from sputum of a man infected in laboratory in 1938. Following recovery of this patient from acute infection, the virus was isolated from his sputum at intervals during the following 8 years.

11. H-We.—Isolated in 1947 from patient. Nc history of avian contact was given.

12. H-Se —Isolated in 1947 from patient who gave history of contact with linnets.

13. H-Ga.—Isolated in 1946 from patient who gave no history of avian contact.

14. H-Ca.—Isolated in 1947 from laboratory worker in contact with the Endo pigeons listed below.

15. H-Cl.—Isolated in 1943 from patient who gave history of contact with pigeons.

16. Meningopneumonitis (Cal 10).—Isolated in 1938 by Francis and Magill (Turner and Fleming, 1939; Beck et al, 1944). Received from Dr. G. Meiklejohn, 1948.

17. Pasadena parakeet.—Isolated in 1947 from parakeet.

18. Washington parakeet.—Isolated in 1946 from parakeet.

19. Seattle parakeet.—Isolated in 1948 from parakeet.

20. Schaefer parakeet.—Isolated in 1946 from parakeet.

21. 6BC.—Isolated in 1941 from parakeet.

22. Detroit parakeet.—Isolated in 1946 from parakeet.

23. P-Endo-1.
24. P-Endo-3.
25. P-Endo-4.
26. P-Endo-D.

Isolated in 1947 from immature pigeons in a single flock.

27. P-Au 46.—Isolated in 1948 from pigeon.

28. P-207.—Isolated in 1943 from pigeon.

29. Petaluma.—Isolated in 1946 from duck.

30. Long Island.—Isolated in 1945 from duck.

31. Friend.—Isolated in 1947 from chicken.

32. New York dove.—Isolated in 1947 from dove.

33. Washington canary.—Isolated in 1946 from canary.

34. Feline pneumonitis.—Isolated by Baker (1942) from cat. Received from Dr. Baker in 1948.

35. Bar Harbor mouse.—Isolated in 1942 from mice.

36. de Burgh mouse.—Isolated by de Burgh et al (1945) from mice. Received from Dr. de Burgh in 1948

37. Mouse pneumonitis (Nigg).—Isolated from mice by Dr. Clara Nigg (1942). Received from Dr. Nigg in 1947.

38. Lymphogranuloma venereum-1.—Received from Dr. G. Rake in 1948.

39. Lymphogranuloma venereum-2.—Received from Dr. G. Meiklejohn, 1948.

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THE TOXINS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP OF AGENTS.

II. EFFECT OF AUREOMYCIN AND PENICILLIN UPON THE TOXINS OF PSITTACOSIS VIRUSES

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In a previous publication (Manire and Meyer, 1950), the toxicity for mice of 39 strains of viruses in the psittacosis-lymphogranuloma group was reported. Following the intravenous injection of yolk sac suspensions into groups of mice, two-phase death curves were plotted from the death rates of mice injected with the Louisiana, Illinois, meningopneumonitis and feline pneumonitis viruses, but definite two-phase curves for the remaining strains were not demonstrated.

Rake and Hamre (1944) studied the effect of sulfamerazine on the toxins of the viruses of lymphogranuloma, mouse pneumonitis and feline pneumonitis. The treatment of mice with this drug did not affect the toxins of these agents, and similar toxin titers were obtained when virus suspensions were tested in control and drug-treated mice. In mice injected with the mouse pneumonitis virus, however, deaths from infection did not occur in the drug-treated group. The drug had no effect on the feline pneumonitis toxin or on infections by this agent. As the agent of lymphogranuloma venereum does not infect mice when injected intravenously, sulfonamides had no effect on the death curve of mice injected with it.

The effectiveness of penicillin in treating psittacosis infections has been amply confirmed by Heilman and Herrell (1944a, 1944b), Parker and Diefendorf (1944), Bedson and May (1945), Meiklejohn et al (1946), Wiseman et al (1946), Meyer and Eddie (1947) and others.

According to Wong and Cox (1948) aureomycin is also effective against experimental infections of mice and of embryonated eggs by the lymphogranuloma venereum and psittacosis viruses.

On the basis of the reported effectiveness of penicillin and aureomycin in psittacosis infection, it appeared possible that deaths of mice due to toxin and those due to infection following intravenous inoculation of toxic virus suspensions might be definitely distinguished by treating mice with these drugs. Such a differentiation was considered of especial value in the case of those viruses which do not show the diphasic death curve. Three groups of viruses were selected for testing, namely, the Louisiana and feline pneumonitis (Baker) strains which show diphasic death curves in mice; the S-F virus, which is toxic but not infectious for mice by the intravenous route; and the P-Endo-1, H-Se, H-Mu 46, Pasadena parakeet and P-207 strains, which are toxic and infectious for mice by the intravenous route but produce single phase death curves.

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Experimental technique.—Toxic yolk sac suspensions of viruses were prepared and tested for toxicity in mice as previously described (Manire and Meyer, 1950). In each case the toxin was titrated in treated and untreated mice, and the number of deaths was recorded at 12-hour intervals.

In testing the effect of aureomycin, mice were given 0.5 mg of the drug in 0.5 ml of saline at 12-hour intervals. The effect of penicillin was tested by diluting procaine penicillin G in peanut oil in a beeswax and peanut oil suspension, and mice were given 5000 units per day in one subcutaneous injection of 0.1 ml. This dosage level was selected following a preliminary experiment with the Louisiana virus in a group of mice treated with 1000, 5000 and 10,000 units of penicillin per day. Similar results were obtained with each dosage level. Two to three weeks after the injection of toxin, the surviving mice were sacrificed and examined for evidence of infection. In certain cases, organ suspensions from these mice were subinoculated into embryonated eggs and mice to determine the incidence of carriers.

The Louisiana, S-F and feline pneumonitis strains.—Toxic suspensions of these three viruses were prepared, and each was titrated in normal mice and in mice treated with both penicillin and aureomycin.

The penicillin-treated mice injected with the Louisiana toxin received 4 subcutaneous injections of 5000 units each of the drug at 24-hour intervals, beginning 5 hours prior to the injection of the virus suspension. The aureomycin-treated group received 11 intraperitoneal injections of the drug, 0.5 mg each, at 12-hour intervals, beginning 24 hours prior to virus injection.

Penicillin-treated mice injected with the S-F and feline pneumonitis viruses received 5 injections containing 5000 units of penicillin each 14 hours, at 2 hours preceding the virus injection, and at 3 24-hour intervals after challenge. The aureomycin-treated mice received 8 injections of 0.5 mg each at 12-hour intervals beginning 14 hours before the virus injections.

The number of mice dying during each 12-hour period was recorded, and the results are shown in table 1. Figure 1 shows a graphic representation of these results and permits comparison of the death curves produced in control and drug-treated mice.

TABLE 1.—*The effect of aureomycin and penicillin in vivo on the toxicity and infectivity of the Louisiana, S-F and feline pneumonitis viruses in mice.*

Virus	Dilution of virus	Controls	Mice treated with	
			Aureomycin	Penicillin
Louisiana	1:40	12, 12, 12, 12, 12, 12		12, 12, 12, 12, **96**
	1:80	12, **36, 36, 48, 60**		12, 12, **96**, S, S, S
	1:160	**72, 96, 96, 96, 120, 120**		>**120**, S, S, S, S
Louisiana	1:10	12, 12, 12, 12, 12	12, 12, 12, 12, 12	
	1:20	12, 12, 12, 12, 12	12, 12, **24, 24, 24**	
	1:40	12, 12, 12, **24, 24**	**24, 24, 24, 24, 24**	
	1:80	**48, 60, 72, 96, 120**	S, S, S, S, S	
S-F	1:10	12, 12, 12, 12, 12	12, 12, 12, 12	12, 12, 12, 12
	1:20	12, **24, 36**, S, S	12, 12, 12, **24**	12, 12, 12, 12, S
	1:40	>**120**, S, S, S, S	S, S, S, S	S, S, S, S, S
Feline pneumonitis	1:10	12, 12, **48, 60, 72**	12, 12, 12, **24**	**24, 24**, S, S, S
	1:20	**24, 60**, >**120**, S, S	S, S, S, S, S	S, S, S, S, S
	1:40	**60**, >**120**, >**120**, S, S	S, S, S, S, S	S, S, S, S, S

Dead mice were collected at 12-hour intervals for 3 days, and daily thereafter.
Mice surviving for 21 days are indicated by "S." The numbers indicate the hours after injection in which the animal was found dead. Mice dying after 24 hours (presumably from infection) are indicated by bold-face type.

It is evident in the above table that these drugs had no effect on the toxins of the three viruses under test, and similar toxin titers were obtained in both drug-treated and control mice. Mice were protected against infection with Louisiana and feline pneumonitis viruses. As the S-F virus is not infectious for mice when injected intravenously, no effect of drug treatment was apparent. In the S-F control mice, the two mice dying 36 hours and 144 hours, respectively, after injection showed no gross anatomical evidence of infection by this virus, and elementary bodies were not observed in organ impression smears.

Penicillin appears to be less effective against the Louisiana virus than does aureomycin, as some penicillin-treated mice died after treatment was stopped. Mice that received aureomycin and did not die during the first 24 hours all survived for 21 days. These mice were sacrificed and all were found to have enlarged spleens with no visible lesions. The spleens were pooled, ground in a Waring Blendor, and subinoculated into yolk sacs of embryonated eggs. The eggs became infected, showing that these survivors were carriers.

No deaths occurred between the 2nd and 21st days in the penicillin- and aureomycin-treated mice receiving feline pneumonitis virus. Inoculations of spleen suspensions from the control and aureomycin-treated mice into yolk sacs of embryonated eggs gave negative results, indicating that the survivors were

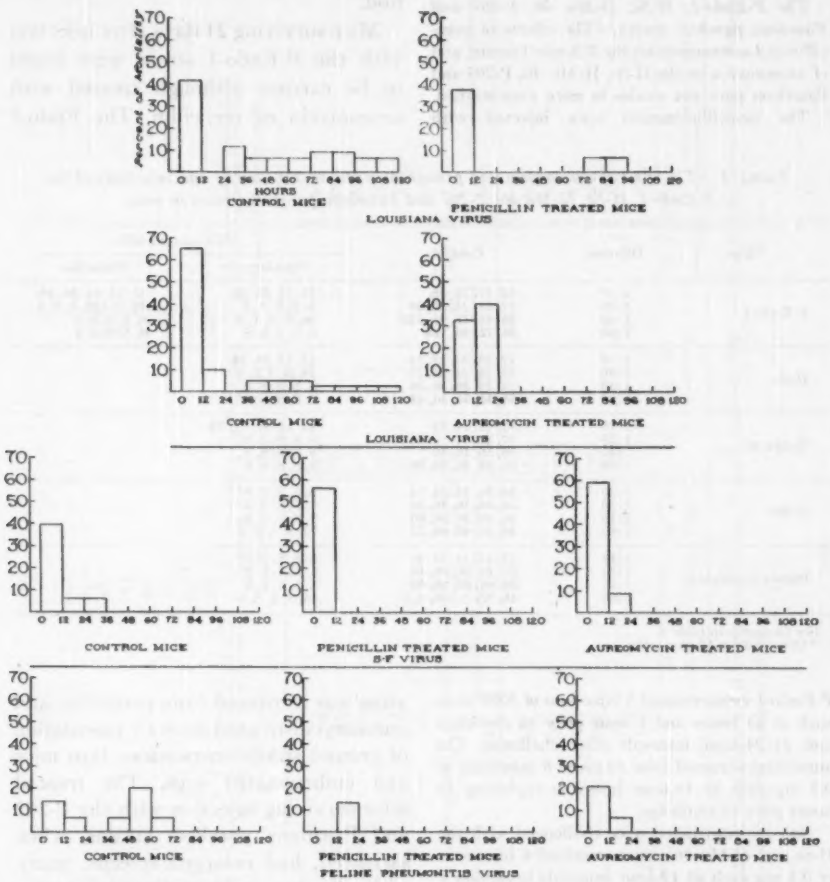

FIG. 1.—Mortality rates of control and treated mice during 12-hour intervals after injection of toxic suspensions of Louisiana, S-F and feline pneumonitis viruses.

not carriers of the virus. Survivors of the S-F virus inoculation were likewise found to be free of infection, as determined by inoculation of spleen suspensions into embryonated eggs.

The P-Endo-1, H-Se, H-Mu 46, P-207 and Pasadena parakeet strains.—The effects of penicillin and aureomycin on the P-Endo-1 strain, and of aureomycin on the H-Se, H-Mu 46, P-207 and Pasadena parakeet strains in mice were studied.

The penicillin-treated mice injected with P-Endo-1 virus received 5 injections of 5000 units each at 13 hours and 1 hour prior to challenge and at 24-hour intervals after challenge. The aureomycin-treated mice received 8 injections of 0.5 mg each at 12-hour intervals beginning 13 hours prior to challenge.

Aureomycin-treated mice challenged with the H-Se and H-Mu 46 viruses received 4 injections of 0.5 mg each at 12-hour intervals beginning 2 hours before challenge. The treated mice injected with the P-207 and Pasadena viruses received 6 injections of 0.5 mg of aureomycin each at 12-hour intervals beginning 14 hours prior to challenge.

Test results are shown in table 2 and presented graphically in figure 2.

In the case of the P-Endo-1, H-Se and H-Mu 46 strains, toxin titers in treated mice were about one-half those of control mice. In the mice injected with P-207 and Pasadena parakeet viruses, only 2 deaths occurred among treated mice during the 21-day observation period.

Mice surviving 21 days after injection with the P-Endo-1 strain were found to be carriers although treated with aureomycin or penicillin. The Endo-1 virus was recovered from penicillin- and aureomycin-treated mice by inoculation of ground spleen suspensions into mice and embryonated eggs. The treated mice surviving injection with the P-207 and Pasadena parakeet strains, when sacrificed, had enlarged spleens, many with discrete lesions. Carrier studies were not made on the mice surviving injection with the H-Se and H-Mu 46 strains.

In experiments using the H-Se, H-Mu 46, P-207 and Pasadena parakeet strains, all control and treated mice dying during the first 36 hours were autopsied. All of these agents produced very similar

TABLE 2.—*The effect of aureomycin and penicillin in vivo on the toxicity and infectivity of the P-Endo-1, H-Se, H-Mu 46, P-207 and Pasadena parakeet viruses in mice.*

Virus	Dilution	Control	Mice treated with	
			Aureomycin	Penicillin
P-Endo-1	1:10	12, 12, 24, 36	24, 24, 36, 36	12, 12, 24, 36, 36
	1:20	12, 24, 24, 36, 36	S, S, S, S, S	72, >120, S, S, S
	1:40	36, 48, 48, 96, 120	96, S, S, S, S	S, S, S, S, S
	1:80	36, 72, 96, 120	S, S, S, S, S	S, S, S, S, S
H-Se	1:10	12, 24, 24, 24, 24	12, 12, 24, 24	
	1:20	24, 24, 24, 24, 24	24, S, S, S, S	
	1:40	24, 36, 36, 36, 36	S, S, S, S, S	
	1:80	36, 36, 36, 36, 48	S, S, S, S, S	
H-Mu 46	1:10	12, 12, 24, 24	12, 12, 12, 12, 24	
	1:20	12, 24, 24	S, S, S, S, S	
	1:40	36, 36, 36, 48	S, S, S, S, S	
	1:80	36, 36, 36, 36, 36	S, S, S, S, S	
P-207	1:10	24, 24, 24, 24, 24	S, S, S, S, S*	
	1:20	36, 36, 36, 36, 36	S, S, S, S, S	
	1:40	36, 36, 36, 36, 60	S, S, S, S, S	
	1:80	36, 36, 48, 60, 72	72, S, S, S, S	
Pasadena parakeet	1:10	12, 12, 24, 24, 36	S, S, S, S, S*	
	1:20	24, 24, 36, 36, 48	S, S, S, S, S	
	1:40	36, 60, 60, 60, 60	S, S, S, S, S	
	1:80	48, 72, >120, S, S	36, S, S, S, S	

See footnotes in table 1.
* Observed for 14 days only.

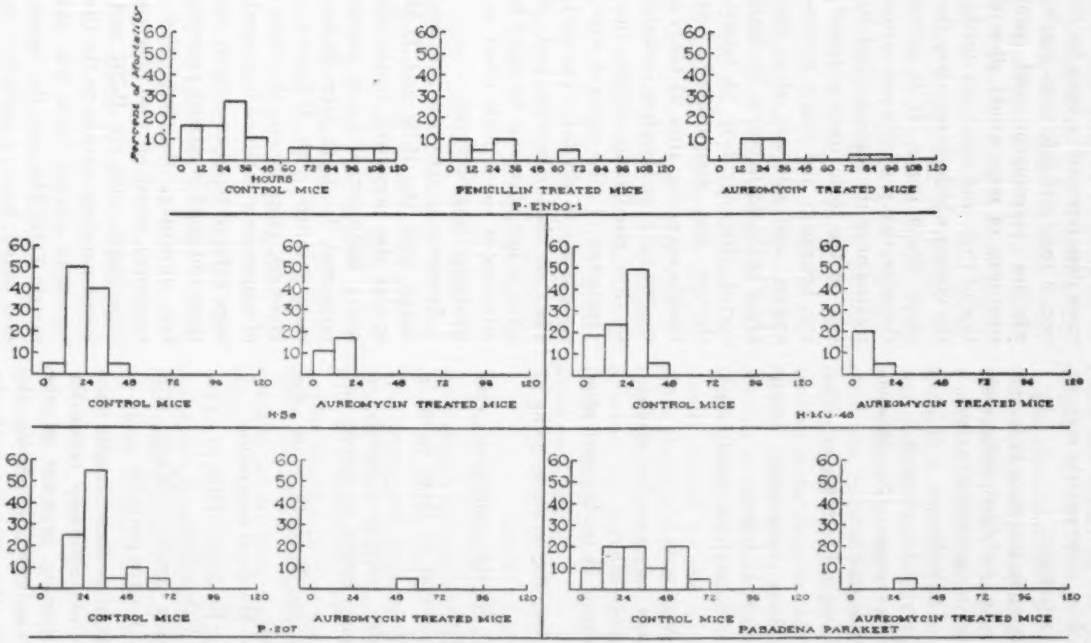

FIG. 2.—Mortality rates of control and treated mice during 12-hour intervals after injection of toxic suspensions of P-Endo-1, H-Se, H-Mu 46, P-207 and Pasadena parakeet viruses.

gross pathological changes in injected mice, and differences were not seen in the dead control and treated mice. The most striking observations may be summarized as follows:

1. Those dying in less than 12 hours:
   Lungs.—Red, enlarged, congested; clear fluid in the pleural cavity.
   Spleen.—Little change.
   Liver.—Very pale, yellowish.
   Impression smears.—No elementary bodies visible.
2. Those dying in 12 to 24 hours after injection:
   Lungs.—Less congestion, more fluid than at 12 hours.
   Spleen.—Normal size, usually dark red.
   Liver.—Very pale.
   Impression smears.—No elementary bodies visible.
3. Those dying 24 to 36 hours after injection:
   Lungs.—Similar to those dying in 24 hours.
   Spleen.—Slightly enlarged, very dark color.
   Liver.—Similar to those dying in 24 hours.
   Impression smears.—Clusters of elementary bodies in spleen and liver.
4. Those dying 36 to 48 hours after injection:
   Lungs.—Marginal congestion.
   Spleen.—Enlarged, dark.
   Liver.—Pale yellow.
   Impression smears.—Many cells filled with virus particles.

It appears from these results that toxic deaths of mice may occur for 36 hours following injection of the P-Endo-1 virus but only during the first 24 hours after injection with the H-Se and H-Mu 46 strains.

DISCUSSION

It was the original purpose of these experiments to determine whether the diphasic death curve produced by the Louisiana and feline pneumonitis viruses does represent a basis for distinction of toxic and infectious deaths, and whether aureomycin and penicillin treatment of mice would allow definition of toxic and infectious deaths with the viruses which do not show the two-phase death curve. It is significant, therefore, that penicillin and aureomycin treatment of the test mice had little effect on the toxin titers obtained with the Louisiana and feline pneumonitis strains, and did not alter the first phase in the death curve. No deaths occurred after the first 24 hours until therapy was discontinued, however. Deaths occurring after 24 hours in untreated mice are therefore probably due to virus proliferation within the host. Treatment of mice injected with toxic suspensions of the S-F virus had little effect on the toxin titer, and, as this virus is not infectious for mice by the intravenous route, little effect of drug treatment was observed.

Aureomycin treatment of mice injected with the H-Se and H-Mu 46 strains also prevented deaths after 24 hours. Both penicillin and aureomycin prevented the death after 36 hours of mice injected with P-Endo-1 virus. However, in each case, the toxin titers of suspensions obtained in treated mice were reduced to approximately one-half those obtained in control animals. It is not surprising, therefore, that drug treatment saved all test mice used in experiments with the P-207 and Pasadena parakeet viruses, as the titer obtained in control mice was approximately 1:10. This was the lowest dilution tested; hence a reduction in titer by one-half would result in survival of all treated mice.

Impression smears from the lungs, spleens and livers of the mice dying

within 24 hours after injection with the H-Se, H-Mu 46, P-207 and Pasadena parakeet strains showed no evidence of virus propagation, i.e., elementary bodies were not seen. Smears from control mice dying in the 24- to 36-hour interval, however, showed considerable numbers of infected cells, and those taken from mice dying in the 36- to 48-hour interval showed many cells distended with virus particles. The fact, however, that during the first 24-hour period more deaths occurred in the untreated groups of mice than in the mice treated with aureomycin suggests that some virus proliferation did take place during this period in the absence of the drug, although proliferation was not evident by microscopic examination. Such rapid virus proliferation would explain the absence of two-phase death curves in untreated mice. The actual toxicity of the inoculum consequently would account for the deaths during the first 24 hours in the treated groups of animals.

A further pertinent observation is that toxins of the Louisiana, S-F and feline pneumonitis virus strains generally are more rapidly lethal than toxins of the above group; this suggests either characteristic differences in the speed with which the toxins act or differences in the rate of release of toxins from the virus particles.

Rake and Jones (1944) reported that the toxic components of these viruses cannot be separated from the elementary bodies by filtration or centrifugation. The extreme lability of the toxins, which are destroyed at incubator temperatures in a short time, and their detoxification by formalin without loss of antigenicity are not generally characteristic of bacterial endotoxins, however. In several characteristics, such as lability, detoxification, rapid effect in mice, etc., these toxins resemble the toxins found in studies with some of the rickettsiae, which, as Doerr (1948) suggests, are similar to the bacterial exotoxins.

Therefore, the rate of release of the toxic component would directly affect the rapidity with which injected mice would die. On this basis it can be hypothesized that the toxicity of the Louisiana, S-F and feline pneumonitis virus suspensions was high, and this component was rapidly released. Additional deaths would then not occur until propagation of the virus was established and further toxin proliferated. The toxin content of the suspensions of the other viruses could be considered as low or closely bound to the virus particles. After the injection of such suspensions into mice, some deaths would occur as a result of the initial toxicity, but further deaths would depend upon rapid virus proliferation.

SUMMARY

The in vivo effects of aureomycin on 8 virus strains and the effects of penicillin on 4 of these strains were studied. Treatment with these drugs protected against death from infections induced with all 8 strains of virus.

The toxicity of the Louisiana, S-F and feline pneumonitis virus strains was not affected by drug treatment of test mice.

The toxins of those viruses which do not show a time lapse between toxic and infectious deaths in mice were affected by these drugs, as shown by reduced titers obtained in drug-treated mice.

Possible explanations for the differences in the drug action on these two groups of toxins are offered.

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THE TOXINS OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP OF AGENTS.

III. DIFFERENTIATION OF STRAINS BY THE TOXIN NEUTRALIZATION TEST

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The close antigenic relationship between the various agents in the psittacosis-lymphogranuloma group as shown by the complement fixation test has been amply demonstrated (Rake, Eaton and Shaffer, 1941; Eaton, Martin and Beck, 1942; Thomas and Kolb, 1943; Smadel, Wertman and Reagan, 1943; and others). In view of this close relationship among its members, many studies have been conducted to find a basis for differentiation and identification of the various strains which comprise the group. Methods based on determination of the infection spectrum of these agents in various experimental animals have been reported by Pinkerton and Moragues (1942) and Beck, Eaton and O'Donnell (1944). Cross immunity tests in mice have been used by Beck and Eaton (1942), Eaton et al (1942), Beck et al (1944), Zichis and Shaughnessy (1945), Larson and Olson (1946), and Wagner, Golub and Andrew (1949). The results of these studies indicate that the S-F, Illinois and Louisiana pneumonitis strains are relatively specific, strains of psittacine origin are less specific, and that the meningopneumonitis and pigeon viruses have relatively broad antigenic structures.

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The absence of potent neutralizing antibodies in the serums of convalescent patients and hyperimmunized animals has been noted by various investigators (Rivers and Berry, 1931a, 1931b; Bedson, 1933; Francis and Magill, 1938; Lazarus and Meyer, 1939; and Rudd and Burnet, 1941). However, Hilleman (1945) prepared neutralizing antibodies of high titer and sharp specificity by intraperitoneal injections of these viruses into chickens. When antiserums against the meningopneumonitis, lymphogranuloma venereum and mouse pneumonitis viruses were used in neutralization tests, the meningopneumonitis and pigeon viruses were found to resemble each other but to differ from the other agents tested. Antiserums against the lymphogranuloma and mouse pneumonitis viruses were specific. St. John and Gordon (1947) prepared additional antiserums, and the antiserum they prepared against the feline pneumonitis virus was also specific. They proposed that this test be used for classification of the group.

The complexity of the methods presently used to differentiate these strains is amply demonstrated in the review by Cox (1947), in which differences among the strains are discussed on the bases of source, cross immunity tests, pathogenicity for various animals, resistance to drugs, virus neutralization tests and toxin neutralization tests. The value of simplified procedures of differentiation is evident.

An additional means of studying the immunological relationships of these viruses was brought into use with the

discovery by Rake and his associates that yolk sac suspensions of viruses of this group are toxic (Rake and Jones, 1944; Hamre and Rake, 1944). Specific toxin-neutralizing antibodies were prepared by injecting toxin or toxoid into rabbits and chickens. Antitoxic serums against the viruses of meningopneumonitis, feline pneumonitis, mouse pneumonitis and lymphogranuloma venereum were prepared, and the sharp specificity of these antibodies was shown by mouse tests. Specific neutralization of the toxins was brought about either by incubation of the serum-virus suspension prior to inoculation into mice, or by passively immunizing mice prior to injection of the virus. These experiments, therefore, suggested that such techniques might be useful in differentiating the members of this virus group. However, the four viruses chosen for the above studies are rather easily distinguished from one another by pathogenicity, tissue tropism and cross testing in immunized mice. It should be expected, therefore, that if specific neutralizations can be demonstrated by this test, antitoxic serums prepared against these four very different viruses should show great specificity.

It was the ultimate aim in the experiments reported here to determine the specificity of this reaction among groups of similar strains and, further, to determine whether the technique might be used in the definitive differentiation of strains having various origins. It was also felt that such a study might contribute further information about the immunological relationships and epidemiology of this group of infectious agents.

MATERIALS AND METHODS

Virus strains.—The strains tested by this technique were selected from the toxin-producing members of the group described previously (Manire and Meyer, 1950). Nearly all strains which produced sufficient toxin to kill mice in 24 hours in a dilution of 1:10 or greater were tested.

Production of antiserums.—Antitoxic serums for various viruses were prepared by the injection of virus suspensions into both rabbits and chickens. Only a few rabbits were immunized, and as the rabbit serums appeared to have lower titers and less stability than the chicken serums, the latter were afterwards used exclusively. The results with chicken serums alone are included in this report. It was also felt that chickens would produce few or no antibodies against normal yolk sac proteins. In a number of early neutralization tests using serums from rabbits immunized with yolk sac suspensions, the serum-yolk sac suspensions caused death in most mice within one or two minutes. Although not proved, it appeared that these deaths might have been due to the injection of particulate matter possibly resulting from a precipitin reaction between the serum antibodies and yolk sac antigens. Such difficulties were seldom encountered when chicken serums were used.

Healthy, young, mature cockerels were selected for immunization. Fresh toxic 20% suspensions of infected yolk sac were used as antigens. Two schedules of immunization were used. The first method, used in the initial studies, consisted of the injection of 1 ml of a 20% toxic yolk sac suspension into each of 2 chickens every second day for a series of 6 injections. After a rest period of 2 to 4 weeks, a second series of injections was given. All injections were made by the intraperitoneal route.

Several variations of this method were soon found necessary. The initial injection of the Louisiana virus suspension into two roosters was extremely toxic and killed the birds within a short time. Other roosters died of shock within a few minutes after injection. Also, it was found that shorter series of injections gave rise to equally good serum titers. Consequently, the second method was used in the preparation of most serums to be reported here.

For most virus strains used in immunizations a toxoid was prepared. To 20% suspensions of infected yolk sacs 0.2% formaldehyde was added and the mixture incubated for 24 hours at 37 C. After inactivation the toxoids were stored at 5 C. until used. To eliminate deaths from shock or emboli, the intramuscular route of injection was chosen. The general schedule of immunization was as follows: (1) One ml of a 20% yolk sac suspension (toxoid or toxin) was injected on each of 3 consecutive days. (2) After 5 days, 1 or 2 ml of a toxic yolk sac suspension (20%) were injected on each of 3 consecutive days. (3) After 5 days, 2 ml

of yolk sac suspension were injected on each of 3 consecutive days. (4) After 2 to 4 weeks a single booster injection of 1 or 2 ml was given.

Six or 7 days after the last injection a trial bleeding was made from the wing vein, and the serum tested. Birds having satisfactory titers were bled out from the heart, and the serums collected after clot shrinkage and centrifugation. If titers were unsatisfactory, the birds were given an additional series of 3 daily injections, and bled out after one week. This procedure produced several satisfactory antiserums. Only in an occasional chicken did the injection of active virus suspensions result in noticeably harmful effects, although some weight loss was common.

Toxin-neutralizing serums were produced in chickens against the toxins of the Louisiana, S-F, H-Se, H-We, H-Mu 46, Cal 10 (meningopneumonitis), P-Endo-1, P-Endo-3 and feline pneumonitis virus strains.

This selection of strains is admittedly not ideal. Additional avian strains were used in attempts to produce other antiserums, but the serums either had very low titers or the titers dropped rapidly on storage. Unfortunately, antiserums against the toxins of strains isolated from parakeets, other pigeons, etc. could not be prepared, but due to time and space requirements the number of serums produced was limited.

All bleeding was done aseptically, and the collected serum was stored at 5 C in 10 ml volumes in acid-cleaned sterile test tubes fitted with rubber stoppers. Preservative was not added to the serum, and little difficulty with contamination was encountered. Prior to use in cross neutralization tests, all serums were titrated for toxin-neutralizing antibodies against the homologous virus strains.

Neutralization tests.—Toxic yolk sac suspensions used in neutralization tests were prepared as previously described. The toxicity of each suspension was titrated in twofold dilutions in mice prior to use in neutralization tests.

In titrating homologous antiserums, twofold dilutions of the serums beginning at 1:2.5 were prepared in 2 ml amounts in small test tubes. To each dilution were added 2 ml of toxin suspension, containing, if possible, 4 to 8 lethal doses of toxin per ml. Each tube was fitted with a rubber diaphragm stopper, shaken well to mix, and left standing at room temperature for 2 hours with occasional shaking. Then 0.5 ml of each suspension was injected into each of 5 or 6 mice. The toxicity of the virus suspension was titrated at the same time in normal chicken serum. The number of deaths was recorded at regular intervals.

As equal amounts of toxin and serum dilution were mixed for each test, each of these was diluted an additional two times, and each mouse was tested with 0.5 ml, containing theoretically one or two lethal doses of toxin in diluted antiserum.

In cross protection tests, the various serums were diluted to contain 2 to 4 times that amount of serum required to neutralize 2 to 4 lethal doses of homologous toxin. As the serums were more stable than the toxins, each toxin was tested with all the available serums in one test. Two ml of the appropriate dilutions of the serums were placed in small tubes, and an equal amount of toxic yolk sac suspension containing 4 to 8 lethal doses per ml was added to each and to 2 ml of normal serum (1:2.5) for control.

Each tube was fitted with a rubber diaphragm stopper and shaken lightly but thoroughly to mix. At intervals the tubes were inverted for thorough mixing, and after 2 hours groups of 5 or 6 mice were each injected with 0.5 ml of each serum-virus mixture. Each mouse thus received a calculated 1 or 2 lethal doses of toxin. In each test, however, a toxin titration in normal chicken serum was done, and the actual lethal doses per mouse were calculated from this.

Mice weighing 12 to 18 g were used in these experiments, but with each virus tested, only mice of uniform size were selected.

Shortly after injection, mice that died from shock during the injection were removed. All later deaths were recorded at regular intervals.

RESULTS

For convenience, the viruses tested have been divided into groups on the basis of source.

Not all the strains were tested against every antiserum. Some of the serums were used up, and the problem of mouse supply was critical. However, as the H-Se and H-We antiserums and the P-Endo-1 and P-Endo-3 antiserums gave almost duplicate results, use of only one serum from each group was considered sufficient. Two end points were used in recording neutralization. When all controls died within 24 hours, the 24-hour end point was selected. Otherwise an end point 30 to 36 hours after injection was chosen.

The viruses isolated from mammalian

sources.—Toxic suspensions of various viruses from human and other mammalian sources were tested against the available antiserums. The results of these tests are summarized in table 1.

H-Se antiserums afforded partial protection against the Illinois toxin, but only the H-Mu 46 antiserum protected one-half of the test mice.

The H-Se, H-We, H-Mu 46, H-Br,

TABLE 1.—*Results of toxin neutralization tests with strains isolated from mammalian sources.*

Strain	Approximate lethal dose per mouse	Antiserums									
		La	S-F	H-Se	H-We	H-Mu 46	Meningopneumonitis (Cal 10)	P-Endo-1	P-Endo-3	Cat	Normal Control
Louisiana	2							5/5		5/5	4/4
	2	0/5*					6/6	4/6	4/6		5/6
	4	0/6	6/6	6/6		6/6					4/4
S-F	2			4/5		0/5				5/5	5/5
	2		0/6	4/6	5/6	0/6					5/5
	2	6/6					6/6	6/6	6/6		6/6
M.D.	1	4/6	0/6	4/5		4/4		4/5			5/5
	4	6/6	3/6	6/6		6/6	6/6	6/6			4/4
H.P.	2	5/5	0/4	5/5		4/5		3/5			4/4
Illinois	1		4/5	1/5		1/5				5/5	5/5
	2–4	5/5	5/5	4/5		4/5					5/5
	1	4/4	2/4	2/2		0/3	3/3	3/4			4/4
H-Se	1			1/6	0/6	0/5	2/6	4/6			5/5
	1–2	5/5					1/5	5/5	5/5		5/5
	1		6/6	0/6	0/6	0/6					4/4
H-We	1–2	5/5					3/5	5/5	5/5		5/5
	1		4/6	0/6	0/6	0/6					3/4
H-Mu 46	2	6/6	6/6	2/6	1/6	0/6	2/6	4/6			4/4
H-Jo	1	5/6		0/6	0/6	0/6					5/5
	2–4	5/6	6/6	3/6		3/6		5/6			4/4
H-Pa	2	5/6	6/6	4/6		1/6	3/6	6/6			5/5
H-Br	1–2	5/5	6/6	0/6		0/6	2/6	5/5			4/4
H-St	2–4	6/6	5/5				3/6	5/5			6/6
	2	5/5					1/5	4/5	5/5		5/5
H-Cl	2		4/4	0/4		0/5		4/4			3/3
	1	6/6					0/6	3/6	4/6		6/6
Meningopneumonitis (Cal 10)	1		5/6	2/6		0/6	0/5	1/6			4/4
	2	6/6					0/6	0/6	1/6		6/6
Feline pneumonitis	1	4/6	3/6	4/6		6/6					5/5
	2	6/6	6/6				6/6	6/6			6/6
	1									0/30	4/5
Bar Harbor mouse	2	6/6	6/6	0/5	0/6	5/6	3/6	6/6	6/6		4/4

* Number of mice dying during observation period/number inoculated.

As shown by these results, the Louisiana toxin was specific, being neutralized only by the homologous antiserum. The S-F, M.D. and H.P. strains showed specific neutralization by the S-F antiserum. (The M.D. and H.P. viruses were isolated from persons infected by contact with the S-F strain.) The H-Mu 46 antiserum gave complete neutralization of the S-F toxin but did not protect mice against the M.D. and H.P. toxins. Some evidence was found that S-F and H-Cl and perhaps the H-Pa and H-St strains react similarly. Evidence of neutralization of the toxins of these strains by the Louisiana, S-F, P-Endo-1 and P-Endo-3 antiserums was not found. The H-Se antiserum failed to neutralize the H-Pa toxin and was not tested against the H-St strain but showed significant neutralization of the other toxins. All strains tested were neutralized by the H-Mu 46 antiserum.

The meningopneumonitis virus toxin

was neutralized not only by the H-Se, H-Mu 46 and homologous antiserums, but also by the P-Endo-1 and P-Endo-3 antiserums.

The specificity of the feline pneumonitis virus toxin is apparent in the above table, as it was neutralized only by the homologous serum. The Bar Harbor mouse strain, however, was acted similarly and were neutralized by the H-Mu 46, meningopneumonitis, P-Endo-1, and, where tested, the P-Endo-3 antiserums. With the exception of the P-Endo-3 strain, the toxins of this group were not neutralized by the Louisiana, S-F, H-Se and H-We antiserums. The H-Se and H-We antiserums neutralized the P-Endo-3 toxin.

TABLE 2.—*Results of toxin neutralization tests with strains isolated from avian sources.*

Strain	Approximate lethal dose per mouse	Antiserums									
		La	S-F	H-Se	H-We	H-Mu 46	Meningopneumonitis (Cal 10)	P-Endo-1	P-Endo-3	Cat	Normal Control
Pasadena parakeet	2									5/5	5/5
	1–2	6/6*	6/6	1/6		1/6	1/6	3/6	6/6		4/4
Washington parakeet	2	6/6	4/6	0/6			0/6	5/6			5/5
Shaffer parakeet	1	6/6	4/5	0/6		0/6	0/6	4/6			5/5
P-Endo-1	1–2		5/6	6/6	6/6	2/6		1/6			4/4
	2	5/5					1/6	0/5	1/4		5/5
P-Endo-3	<1		3/5	0/5		0/5		1/5			2/4
	1	5/6	4/6	2/5	2/5		0/6	3/6	0/5		5/5
	1	4/6					1/6	0/6	0/6		6/6
P-Endo-4	1	4/5	5/5	4/5		1/6	0/5	0/5			3/4
P-Au 46	1	6/6	4/4	3/3		1/3		0/5			4/4
P-207	2	5/5	5/5	1/6		1/5	1/6	6/6			5/5
	2	4/5	4/6	0/6	0/6		0/6	5/6	6/6		5/5
Petaluma duck	1–2	6/6	4/6	0/6	0/6	2/6	1/6	5/6	5/5		5/5
Friend chicken	1–2	5/5	5/5	1/6	0/6	2/6	1/5	6/6	6/6		4/5
Washington canary	2	5/5	5/5	0/6			0/6	3/5			4/4

* Number of mice dying during observation period/number inoculated.

neutralized by the H-Se, H-Mu 46 and meningopneumonitis antiserums.

Strains isolated from various avian sources.—The results of neutralization tests using toxic suspensions of viruses isolated from various avian sources are summarized in table 2.

It is of interest that all three parakeet virus strains and one of the pigeon strains gave similar results, the toxins being neutralized by the H-Se, H-Mu 46 and meningopneumonitis antiserums. These results are comparable to those of various human virus strains shown in table 1.

The P-Endo-1, P-Endo-3, P-Endo-4 and P-Au 46 pigeon virus strains resembled the parakeet virus toxins, being neutralized by the H-Se, H-We, H-Mu 46 and meningopneumonitis antiserums but not by the Louisiana, S-F, P-Endo-1 and P-Endo-3 antiserums.

The toxins of the Petaluma duck, Friend chicken and Washington canary virus strains resembled the parakeet virus toxins, being neutralized by the H-Se, H-We, H-Mu 46 and meningopneumonitis antiserums but not by the Louisiana, S-F, P-Endo-1 and P-Endo-3 antiserums.

DISCUSSION

The results obtained with the toxin neutralization technique are summarized in table 3. For the purpose of comparison, positive neutralization is shown if one-half or more of the mice injected with any serum-virus mixture survived the test period. If less than

one-half of the mice survived, negative results are shown.

On the basis of the results of these experiments, the 27 virus strains tested are arranged into 6 separate specific groups. Unfortunately, additional antiserums were not available for this study. The use of antiserums against viruses isolated from parakeets, pigeons, chickens, etc. might well furnish bases for further differentiation. Definite grouping of these viruses on the basis of the toxin neutralization test is not possible until a large variety of additional antiserums are studied. The specificity of the neutralizations encountered in this study does, however, give significant support for such a classification on a tentative basis.

The toxin of the Louisiana virus and the homologous antiserum were specific; none of the other antiserums neutralized this toxin, and no other toxins were neutralized by the Louisiana antiserum. The toxin of the feline pneumonitis virus was likewise neutralized only by its homologous antiserum, and in the tests which were done, the feline pneumonitis antiserum gave no evidence of neutralizing heterologous toxins.

The toxins of the S-F group of viruses were all neutralized specifically by the S-F antiserum, although the S-F virus also was neutralized by the H-Mu 46 antiserum, which did not neutralize M.D. and H.P. toxins. The Illinois virus was another which appeared to be relatively specific, being neutralized only by the H-Mu 46 antiserum. These results confirm a considerable number of

TABLE 3.—*Summary of the toxin neutralization tests.*

Strain (toxin)	Source	Contact	Antiserums: Louisiana	S-F	H-Se	H-We	H-Mu 46	Meningopneumonitis (Cal 10)	P-Endo-1	P-Endo-3	Feline pneumonitis
Louisiana	Man	?	+	–	–		–	–	–	–	–
Illinois	Man	?	–	–	–		+	–	–		–
S-F	Man	?	–	+	–	–	+	–	–	–	–
M.D.	Man	Man	–	+	–		–	–	–		
H. P.	Man	Lab*	–	+	–		–		–		
H-Pa	Man	Pigeons Chickens	–	–	–		+	+	–		
H-St	Man	Pigeons	–	–				+	–	–	
H-Se	Man	Linnet	–	–	+	+	+	+	–	–	
H-We	Man	?	–	–	+	+	+	–?	–	–	
H-Mu 46	Man	Lab*	–	–	+	+	+	+	–		
H-Jo	Man	Pigeons	–	–	+	+	+		–		
Briggs	Man	Pigeons	–	–	+		+	+	–		
H-Cl	Man	Pigeons	–	–	+		+	+	–	–	
P-207	Pigeon		–	–	+	+	+	+	–	–	
Pasadena parakeet	Parakeet		–	–	+		+	+	+?	–	–
Washington parakeet	Parakeet		–	–	+			+	–		
Schaefer parakeet	Parakeet		–	–	+		+	+	–		
Petaluma duck	Duck		–	–	+	+	+	+	–	–	
Friend chicken	Chicken		–	–	+	+	+	+	–	–	
Washington canary	Canary		–	–	+			+	–		
Bar Harbor mouse	Mouse		–	–	+	+		+	–	–	
Meningopneumonitis (Cal 10)	Human? Ferret?		–	–	+		+	+	+	+	
P-Endo-1	Pigeon		–	–	–	–	+	+	+	+	
P-Endo-3	Pigeon		–	–	+?	+?	+	+	+	+	
P-Endo-4	Pigeon		–	–	–		+	+	+		
P-Au 46	Pigeon		–	–	–		+		+		
Feline pneumonitis	Cat		–	–	–		–	–	–		+

* Laboratory infection.

previous observations on specificity. Larson and Olson (1946) vaccinated mice with psittacosis, meningopneumonitis and Louisiana viruses. Mice vaccinated with the heterologous strains were not protected when challenged with the Louisiana virus, but vaccinations with the homologous strain gave considerable protection. Similar specificity has been shown for the S-F and Illinois strains by Beck et al (1944), Zichis and Shaughnessy (1945) and Wagner et al (1949). In these studies, however, the specificity of the S-F and Illinois viruses is apparent only when these strains are used in immunizing mice for cross challenge, whereas mice immunized with other strains were definitely immune to subsequent challenge with the S-F virus. With one exception, to be discussed later, the toxins of these viruses and their homologous antiserums that have been studied are equally specific; and, if cross reactions are obtained, the cross reactions are reciprocal. Data on virus neutralization with these strains are not available, but the results of St. John and Gordon (1947) indicate that the feline pneumonitis virus is sharply specific.

It is of interest that all of these strains are of mammalian (human, feline) origin, and that none originates from a known avian source. The 3 human strains have been transmitted from man to man by contact, and feline pneumonitis virus probably spreads from cat to cat. This recalls the observation of Meiklejohn et al (1944) that strains of the S-F type may represent viruses which have become adapted to man, meanwhile losing some of their original characteristics. On the basis of the results of the toxin neutralization test, this same speculation would apply likewise to the Louisiana, Illinois and feline pneumonitis strains, which have also evidently become adapted to mammals.

Four of the 5 viruses of pigeon origin tested in the above series, the 3 P-Endo and P-Au 46 strains, were also specific in that they were neutralized by the P-Endo antiserums which failed to neutralize the toxins of other strains. Results obtained with the P-Endo-3 strain offer difficulty in interpretation, as the toxin of this strain was neutralized by the H-Se and H-We antiserums, whereas the toxins of the other 3 strains of this group gave no evidence of such neutralization.

On the basis of neutralization by the H-Se and H-We antiserums, 14 of the tested strains were placed in an additional group. Two other strains may belong to this group, but were omitted as the toxin of one, namely the H-Pa strain, was not neutralized by the H-Se serum, while the other, the H-St strain, was not tested with the H-Se and H-We antiserums. This group of agents is of especial interest because of the variety of sources of its member viruses. In the the other groups containing multiple strains, all of the viruses included were isolated from one species. In this group, however, are 6 viruses isolated from infected patients, 3 isolated from parakeets, and one each of pigeon, duck, chicken, canary and mouse origins. The toxins of all member strains, however, react in a remarkably similar manner, showing, with few exceptions, neutralization only by the H-Se, H-Mu 46, meningopneumonitis (Cal 10), and, if tested, by the H-We antiserums.

Three of the strains, the H-Jo, H-Br and H-Cl, were isolated from patients who gave epidemiological evidence of infections resulting from pigeon contacts. The P-207 strain, isolated from pigeons, also is in this group. On the basis of these results it appears that viruses of pigeon origin are of two

distinct types, as these strains react quite differently from the P-Endo and P-Au 46 strains.

The similarity of the Bar Harbor mouse strain to this large group of strains of avian origin is also of interest. All the other mammalian strains, which appear to be species-adapted on the basis of a chain of transmission within the various species, were significantly specific by the toxin neutralization technique. The report of Rake and Jones (1944) indicates that the mouse pneumonitis (Nigg) virus does give specific reactions in this test. St. John and Gordon (1947) presented evidence that two other strains from mice, the Chicago and Ann Arbor mouse viruses, give specific reactions when tested by the virus neutralization technique. De Burgh and associates (1945), however, isolated from apparently healthy mice a virus which they were unable to distinguish from the psittacosis viruses.

As the H-Pa, H-St, H-Se, H-Jo, H-Br and H-Cl virus strains were isolated from patients who gave epidemiological evidence of contact with various birds, it is not surprising that these strains are similar to avian strains. The infection source of the individual from whom the H-We strain was isolated is unknown, but there is no evidence that man-to-man transfer was responsible.

The H-Mu 46 strain, however, does give further evidence that viruses of this group may upon continuous residence become adapted to a mammalian host. This strain was isolated from a human carrier in 1946, 8 years after acute infection. Virus was isolated from the patient's sputum at regular intervals during this time, and the possibility that he might have become infected with another strain meanwhile is remote (Meyer and Eddie, 1947). Very probably the infection was due to a virus of psittacosis origin, as at that time these strains were being studied almost exclusively at the Hooper Foundation where the patient was employed.

The toxin of the H-Mu 46 strain is neutralized by the same antiserums as are the other members of its virus group. Antiserum prepared against this toxin, however, gave surprising results. It not only neutralized toxins of the homologous group but also toxins of the P-Endo and Au 46, the S-F and the Illinois strains. This was the only instance in which a toxin of one strain and its homologous antiserum did not give corresponding reactions. In the case of the other strains tested, antiserums were specific for toxins of all member viruses of a group.

The neutralization of the S-F and Illinois viruses by the H-Mu 46 antiserum indicates that these two strains are related. Zichis and Shaughnessy (1945) revealed the relationship between these viruses by cross immunity tests. This neutralization would also indicate that the H-Mu 46 virus acquired additional antigens as a result of adaptation to a human carrier.

It should be recalled that according to Beck, Eaton and O'Donnell (1944) and Wagner, Golub and Andrew (1949) the S-F virus produces specific immunity in mice, but strains of various origins produce immunity against subsequent challenge with the S-F virus. However, this relationship evidently does not occur in the toxin neutralization test, as the meningopneumonitis and pigeon virus antiserums did not neutralize the S-F toxin. Additional antiserums prepared against psittacine viruses, if tested for neutralization of the mammalian strain toxins, might show these antiserums to react in a manner similar to the H-Mu 46 antiserum; but in view of the sharp specificity of other antiserums tested in these experiments, this is unlikely.

The meningopneumonitis virus has been placed in between the large group neutralized by the H-Se and H-We antiserums and the group of P-Endo and P-Au 46 viruses. The toxin of this virus is neutralized not only by the homologous antiserum, but also by the H-Se, H-Mu 46, P-Endo-1 and P-Endo-3 antiserums. That this is a specific neutralization is evident in that the antiserum to the meningopneumonitis virus neutralizes the toxins of viruses in both of these groups.

It would appear, on the basis of these tests, that the toxin neutralization technique is of definite value in studies on the antigenic relationships of viruses in this varied group of infectious agents. In view of the wide variety of sources represented in the one large group of viruses discussed above, this technique would generally be of little value in determining the exact source of an unknown virus.

SUMMARY

The production of toxin-neutralizing antiserums by hyperimmunization of chickens and the use of these antiserums in cross toxin neutralization tests were described.

Toxin neutralization tests were made with the toxins of 27 virus strains, and antiserums were prepared for the toxins of the Louisiana, S-F, H-Se, H-We, H-Mu 46, meningopneumonitis (Cal 10), P-Endo-1, P-Endo-3 and feline pneumonitis (Baker) virus strains.

The toxins and antiserums of the Louisiana and feline pneumonitis strains were specific, reacting only in homologous mixtures.

The S-F virus toxin was neutralized by both the S-F and H-Mu 46 antiserums, whereas the M.D. and H.P. toxins were neutralized only by the S-F antiserum.

On the basis of the outcome of experiments utilizing this technique, two additional groups of viruses were described. One group is composed of a large number of viruses from a wide variety of sources. The other group consists of four viruses of pigeon origin.

The question of adaptation of these viruses to mammalian hosts is discussed on the bases of specificity of the Louisiana, S-F, Illinois and feline pneumonitis strains and the heterologous relationship of the H-Mu 46 strain to the S-F and Illinois viruses.

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DENTINAL TUBULES AS A ROUTE FOR THE TRANSMISSION OF POLIOMYELITIS VIRUS AND HORSE SERUM

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The experiments reported here were undertaken to learn whether dentinal tubules may serve as a portal of entry for the transit of poliomyelitis virus and other macromolecular substances from a superficial cavity to the abundant neural and vascular supply of the pulp chamber. These studies were designed to supplement the results of experimental[1] and epidemiological[2–6] investigations which have been offered as evidence that the pulpal chamber of a carious tooth can serve as a portal of entry for poliomyelitis in its transmission by afferent neural fibers to the central nervous system. The experimental test of this route for infection appeared desirable, especially since the assumption by Reese and Frisch[4,5] that dentinal tubules may operate as a direct route from surface cavities to the pulpal cavity led others[6] to investigate the possibility of this extraneural route serving as a portal of entry for poliomyelitis virus.

EXPERIMENTAL

Five young mature monkeys (*Macaca mulatta*) with noncarious teeth were selected. Four of these monkeys, on each of 6 successive days, were made ready for inoculation with poliomyelitis virus, Minnesota 1946 strain, while under chlorylene anesthesia. The tooth was isolated with a rubber dam, a small diamond wheel employed to gain access to the dentin, and a dental bur, size 35, used to establish form and retention to a single cavity in the labial surface two-thirds of the distance from the incisal edge to the gingival crest of different teeth. The depth of the cavity was made to encompass the dental bur and therefore approximated 1 to 1.5 mm. The 6 teeth utilized for inoculation were the 2 central and the 2 lateral maxillary incisors and the 2 central mandibular incisors. Special care was exercised not to encroach upon the pulp. Immediately after preparation, each cavity was inoculated with virus of known infectivity by instilling a drop of a 10% suspension of monkey cord for 10 minutes, at the end of which time the excess viral suspension was removed by absorbent cotton pellets; the cavity was sealed by an amalgam restoration. The tooth and the rubber dam before removal were cleansed with 5% cresol.

The 5th monkey for inoculation with B virus was prepared similarly, except that cavities in 2 central maxillary incisors were made ready in a single day, no additional teeth being inoculated. The animals were observed and the temperatures were recorded daily for 35 days.

Six young virgin guinea pigs under pentothal anesthesia were prepared for inoculation as described above with the exception that single cavities were prepared in the mandibular incisors, one on each of 2 successive days. The inoculum for 3 animals consisted of 0.02 ml horse serum, and for 3 guinea pigs of 0.02 ml egg albumin. Two additional animals were prepared for inoculation by the technique described by Weisberger,[7] in which the cavities were established in the incisal edge of the tooth. These 2 animals also received 0.02 ml horse serum administered with a tuberculin syringe and a 27 gauge needle. All cavities were sealed with zinc oxyphosphate cement. Six additional guinea pigs served as controls.

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Fifteen days after the second treatment of the dental cavities with foreign protein, the guinea pigs were killed by exsanguination, and within 4 hours the uterine musculature from each pig was tested by the Schultz-Dale technique for specific hypersensitivity. Each uterine horn was twice tested for specific reactivity to 0.1 ml of the homologous antigen, and then it was subjected to histamine hydrochloride to learn whether the technique, apparatus, and antigen employed in any given experiment were working satisfactorily.

RESULTS

When the cavities for exposure of dentinal tubules were made in the labial surface proximal to the incisal edge, the inoculation with poliomyelitis virus of 24 cavities in 4 monkeys and with B virus of 2 cavities in one monkey failed to reveal any evidence that dentinal tubules, as measured by infection, may serve as avenues for the transfer of virus from superficial cavities to the pulp chamber. Similarly, the exposure to a foreign protein of dentinal tubules within the 16 cavities which had been prepared in 8 guinea pigs failed, as measured by specific sensitization in 7 animals, to demonstrate that the dentinal tubules may act as a portal of entry for macromolecular substances, such as horse serum protein and egg albumin. Successful sensitization was limited to a single guinea pig which had cavities prepared in the incisal edge of the teeth.

DISCUSSION

The present experimental findings do not support the hypothesis that carious teeth can serve as a portal of entry for either infectious or tissue sensitizing macromolecular substances. Contrary to the assumption by Reese and Frisch[4,5] that dentinal tubules may operate naturally as avenues from superficial cavities for the transit of poliomyelitis virus to the neural plexus in a pulpal chamber, experiments designed to test the permeability of the tubules to neurotropic viruses and to proteinaceous sensitizing agents were uniformly negative when the cavity was situated on the labial surface well removed from the incisal edge. It is of especial interest, however, that the tissues of one of the two guinea pigs which had been prepared as described by Weisberger,[7] when tested by the Schultz-Dale technique, was proved to be sensitized following exposure of dentinal tubules to horse serum. This result was attributed to the difference in host species, since the incisors of rodents erupt continuously to present a tract of pulpal remnants instead of the uniformly calcified matrix in the incisal edges that is characteristic of teeth in primates.

The macromolecular agents were selected so as to have 2 viruses, poliomyelitis and B, of proved affinity for the central nervous system, and 2 proteins, horse serum and crystalline egg albumin, of known efficacy for tissue sensitization. Poliomyelitis virus was selected because of the demonstration by Faber and Silverberg[8] of involvement of the Gasserian ganglia in 7 of 8 fatal human poliomyelitis cases, thus making it apparent that the trigeminal afferents may serve as pathways for transfer of virus from pulpal cavities. Furthermore, Reese and Frisch suggested, on the basis of good anatomical support, that dentinal tubules serve as a route from carious teeth for passage of the virus to the neural plexus in the pulpal cavity. It is of interest in support of this hypothesis that freshly exposed dentin has lymph drainage and open tubules, whereas the tubules in dentin following exposure are sealed.[9–11] It was obvious

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that this hypothesis of Reese and Frisch should be tested by adequate experimentation. B virus was selected because of its great pathogenicity and because of its ability to pass from any peripheral site to the central nervous system. The two agents employed for sensitization are known to effect in minute dose the specific sensitization of guinea pig tissues such that exposure by the Schultz-Dale technique of the excised uterine horns to a shock dose of homologous protein regularly results in a measurable reaction. When none of the 4 agents yielded evidence for transit through the dentinal tubules, whether by way of neural fibrils or lymph channels, it was concluded that there was little probability for superficial carious lesions to serve as a portal of entry for delivery of virus or sensitizing agents by way of the dentinal tubules to the pulpal cavity.

These negative findings make worthy of review the evidence in support of the pulpal cavern as a portal of entry for poliomyelitis virus. Aisenberg and Grubb[2] employed 5 monkeys in their study. These animals were prepared for inoculation with virus (a) by exposure of the pulpal cavity, (b) by removal of the pulp by a broach, or (c) by extraction of teeth, either singly or in combination. Each animal received, in the exposed pulpal cavities of from 4 to 12 teeth, an inoculation of from 0.35 to 0.5 ml of virus in suspensions varying from 20 to 60%. Three of the 5 animals were inoculated on 4 occasions at intervals of from 26 to 66 days between each inoculation; one animal was killed 17 days after a single exposure to 0.3 ml of 40% virus. The 5th animal received in the pulpal cavities of 8 teeth 0.5 ml of 20% suspension. It was asymptomatic for 26 days when the same teeth were given 0.5 ml of 40% virus. Evidence of poliomyelitis infection developed 6 days later, and the monkey was killed 2 days thereafter. This animal showed unequivocal histopathological evidence of poliomyelitis infection. It appears questionable, on the other hand, whether the histopathological evidence of poliomyelitis infection in any of the other animals when killed yielded evidence for specific poliomyelitis infection. These limited findings of Aisenberg and Grubb[2] in support of the pulpal cavity as a route of infection were substantiated by epidemiological studies by 3 groups of workers[2–5] who demonstrated a statistically significant difference in the incidence of clinical poliomyelitis among children with visible exposure of one or more dental pulps as compared with children without such exposure. Contrariwise, 4 other groups of workers[6,12–14] in better controlled studies found no evidence of a difference in the incidence of poliomyelitis between patients with pulpal exposure and the control groups without such exposure. It appears, therefore, that the suggestion that dental pulp might be an important portal of entry for poliomyelitis virus may apply only under circumstances that permit gross contamination of the pulpal cavity.

SUMMARY

An experimental study of dentinal tubules as a route for passage for macromolecular substances from surface cavities to pulpal cavities is reported. The exposure to poliomyelitis virus of 24 cavities in 4 monkeys and to B virus of 2 cavities in one monkey failed to reveal evidence as measured by infection that

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dentinal tubules serve as avennes for the transfer of virus from superficial cavities to the pulp cavern. Similarly, the exposure to a foreign protein of dentinal tubules within 16 cavities that had been prepared in 8 guinea pigs failed, as measured by specific sensitization in 7 animals, to demonstrate that the dentinal tubules may act as a portal of entry for tissue sensitizing macromolecular substances. The successful sensitization of a single guinea pig was attributed to the location of the cavities in incisal edges of teeth of a host species, the guinea pig, which presents a tract of pulpal remnants for conduction of the virus instead of the uniformly calcified matrix characteristic of teeth in primates. The significance of these findings is discussed.

TWO OUTBREAKS OF GASTROENTERITIS APPARENTLY CAUSED BY A PARACOLON OF THE ARIZONA GROUP

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An etiologic relationship between the paracolon bacteria and enteric infections in man has not been clearly established. Such a relationship has been difficult to prove because of the frequency with which these organisms have been recovered from the stools of apparently healthy persons and the lack of adequate methods for distinguishing between different types. Nevertheless, a number of observations[1–6] have been reported indicating pathogenic properties of some strains.

In the Arizona group of paracolon bacteria, the identification of different types has been clarified by Edwards and his co-workers.[7–10] The success of these workers in establishing a satisfactory serologic classification has opened the way for more precise studies relating to the pathogenicity of members of this group.

Most of the cultures of the Arizona group which have been studied thus far were isolated from reptiles and domestic animals, chiefly fowls. Many cultures were obtained by Edwards et al[9,10] and by Hinshaw and McNeil[11–13] during studies of infections among reptiles and turkeys. These studies leave little doubt that some strains are pathogenic for certain animals.

In contrast to the numerous cultures which have been isolated from animals and animal products, comparatively few have been recovered from man. Of 456 cultures studied by Edwards, West and Bruner,[10] only 5 were obtained from human sources. These 5, including one previously reported by Seligmann et al,[14] were isolated from cases of enteric infection in which no other pathogenic forms were recognized. In 1945, Ferris, Hertzberg and Atkinson[15] reported an outbreak of diarrhea in Australia which originally was thought to have been caused by a type of *Salmonella* not previously described. Subsequent study of

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the cultures by Atkinson, however, led her to believe that the etiologic agent was a paracolon of the Arizona group. In July, 1946, Verder et al[16] studied an outbreak of 51 cases of gastroenteritis among 158 student nurses of a Washington, D. C. hospital. A paracolon, identified by Edwards as Ar. 1, 2:1, 2, 5, was isolated from 70 % of the patients whose stools were cultured, but not from any of 16 other students who were not ill. In October, 1948, Buttiaux and Kesteloot[17] reported the isolation of paracolon bacilli from 6 patients—3 with acute enteric disease, 2 with chronic colitis and one with a typhoid-like syndrome. The cultures were reported to be similar in antigenic structure to those described in 1947 by Edwards et al.[10]

Additional evidence is presented in this report with respect to the occurrence of the Arizona group in man. Two small outbreaks of gastroenteritis which occurred almost simultaneously in widely separated areas of the state are described. A member of the Arizona group was associated with both outbreaks under circumstances strongly suggesting an etiologic relationship.

First episode

On June 29, 1949, during the late afternoon, 5 Negro children ranging in age from 6 to 13 years were admitted to an Atlanta hospital with severe gastrointestinal symptoms. The following forenoon a 6th Negro child, age 16, was admitted with similar symptoms. In the first 5 cases the onset occurred about noon on the day of admission, while in the 6th case the symptoms began a few hours later. Each child gave a history of nausea and vomiting followed by diarrhea. In 2 cases chills and headache were mentioned as prominent early symptoms.

Upon admission to the hospital the chief symptoms were fever, diarrhea and dehydration. Stools were watery in character and in one case they were streaked with blood. Temperatures at the time of admission ranged from 103 to 105 F with one reaching 107 F shortly thereafter. Under treatment they dropped rapidly to 101 to 102 F, then gradually returned to normal during the following 2 or 3 days.

Stool specimens from each of the 6 cases yielded almost pure cultures of an organism which was thought to be a member of the *Salmonella* group. The 6 cultures were sent to us for further study. The organisms were gram-negative, motile rods which promptly fermented, with gas formation, dextrose, mannite, maltose, arabinose, rhamnose, trehalose, xylose and sorbitol. They grew on Simmons' citrate agar, produced an abundance of hydrogen sulfide and liquefied gelatin. No reaction was obtained with saccharose, salicin, dulcitol, inositol, adonitol and lactose (after three weeks incubation). Tests for urease and indole production were negative. Although the biochemical reactions indicated that the organisms belonged to the *Salmonella* group, no agglutination was obtained with polyvalent antiserum. The cultures were then sent to Dr. P. R. Edwards, Communicable Disease Center, United States Public Health Service, Atlanta, Georgia, who identified them as members of the Arizona group of paracolon bacteria having the antigenic formula Ar. 10:1, 2, 5.

The source of infection was apparently a picnic lunch which was prepared by the children themselves and eaten at about 5:00 p.m. on the day preceding

16. Verder, E., Bell, J. A., Collins, T. F., Schrieber, J. O. and Brennan, J. F. 1946, Personal communication.
17. Buttiaux, R. and Kesteloot, A. 1948, Ann. Inst. Pasteur, **75**: 379.

the onset of symptoms. The children represented 3 different families, but no illness occurred among the other 12 members who did not attend the picnic.

The principal foods prepared for the picnic and the only ones eaten by all of the children were goose liver sandwiches and ice cream. The goose liver sausage was purchased at a reputable market and portions of the same loaf were eaten by two other families in the neighborhood without causing any illness. Moreover, a remnant of the sausage was cultured and no pathogenic organisms were recognized. Thus, it appears probable that the ice cream served as the vehicle of infection. It was prepared by two of the girls and frozen in a refrigerator, but the history obtained with reference to its preparation failed to provide any clues as to how it might have been contaminated.

On July 13, two weeks after the date of onset, a specimen of blood was collected from each of the children for the purpose of determining their immunologic response to the infection. The culture which had been isolated from one of the patients (D.T.) was used in the preparation of the necessary antigens. After one passage through semisolid agar, a formalized broth suspension was employed as the H antigen. An alchoholic extract was employed as the O antigen. The agglutination reactions against each of the antigens are shown in table 1. All of the serums agglutinated the O antigen in fairly high titer, one showing a reaction in a dilution of 1:2560. The H antigen gave lower reactions, the highest being in a dilution of 1:320.

As a control on the specificity of the antigens, agglutination tests were done on 12 serums submitted for a routine Kahn test. Two of these showed a slight reaction (not over 2 plus) with the O antigen in a dilution of 1:40, but no reactions were obtained with the H antigen. These results are in marked contrast to the uniformly positive reactions obtained with the serums of the patients.

Second episode

This outbreak occurred in one of the wards at Battey State Tuberculosis Hospital and involved 6 white male patients ranging in age from 19 to 45 years. During the night of July 1 they became ill with nausea, vomiting and diarrhea. Temperatures rose abruptly to 101 to 103 F, then gradually returned to normal in 48 to 72 hours. Prostration was a prominent symptom in each case.

Less than 24 hours preceding the onset of symptoms, the 6 patients were served a special food mixture consisting of milk, raw eggs, chocolate and malt powder. The ingredients were mixed in bulk by a kitchen employee during the morning of July 1 and placed in 6 individual containers in the refrigerator. Beginning at 8:00 a.m., portions of the the mixture were served at intervals throughout the day.

TABLE 1.—*Agglutinations with patients' serums—Atlanta outbreak.*

Patients	O Antigen	H Antigen
N.C.	2560	40
J.L.C.	1280	160
M.C.	320	80
B.R.	320	320
A.R.S.	320	80
D.T.	640	40

Figures indicate highest dilution at which agglutination occurred.

The employee who prepared the special feedings was recovering from a diarrhea of three days duration at the time she mixed the ingredients. Her symptoms were not severe and she did not report her illness to the hospital authorities. One other patient in another ward was served a similar mixture the same day. His food was prepared by a different employee, however, and he did not become ill.

Food mixtures similar to the one described above are served from time to time at Battey Hospital to certain patients with advanced lesions who have shown little or no tendency to improve with sanitarium care. On no previous occasion, however, had such feedings been followed by symptoms of food poisoning.

Stool specimens were collected at the hospital and forwarded to us by mail. Persons from whom specimens were collected included the 6 patients with gastrointestinal symptoms, the employee who prepared their feedings, and 14 other patients and employees who had not shown any unusual symptoms. The stool specimens were streaked on MacConkey and bismuth sulfite agar plates, and were planted in tetrathionate enrichment broth which, after overnight incubation, was streaked to bismuth sulfite agar. All of the specimens were negative by direct streaking, but, from 4 of them, suspicious colonies were obtained after tetrathionate enrichment. The colonies were abundant and closely resembled those of *Salmonella typhosa* and other members of the *Salmonella* group, being dark and surrounded by black zones. When fished to Kligler's tubes, abundant hydrogen sulfide was produced.

TABLE 2.—*Agglutinations with patients' serums—Battery Hospital study.*

Patients	Antigen		Stool culture results—Organism isolated
	O	H	
F.W.	160	—	Arizona paracolon
F.L.	160	—	Arizona paracolon
W.W.	80	—	Negative
J.K.	160	—	Negative
D.S.	320	160	Negative
J.R.	160	40	Arizona paracolon

Figures indicate highest dilution at which agglutination occurred.
Dash indicates no reaction at 1:40 dilution.

The biochemical reactions of the 4 isolates were identical with those of the 6 described in the first outbreak. The organisms failed to agglutinate with polyvalent salmonella antiserum. It was observed, however, that agglutination did occur with serum from one of the patients in the Atlanta outbreak. The cultures were then sent to Dr. Edwards for antigenic analysis, and he reported them to be identical with the Arizona strain (Ar. 10:1, 2, 5) which had been isolated from the Atlanta patients.

The 4 positive stool specimens were obtained from 3 of the 6 patients with gastroenteritis and from the employee who prepared their feedings on July 1. Stool specimens from the 14 persons who had not been ill were all negative, indicating that the organism was not widely disseminated at the time of the outbreak.

A specimen of blood was collected from each of the 6 patients 26 days after the onset. Agglutination tests were made using H and O antigens prepared from one of the Battey Hospital cultures (J.R.). The methods employed were similar to those described above except that the culture was passed three times through semisolid agar immediately preceding the preparation of the H antigen. Results of the agglutination tests are shown in table 2. The antibody response shown by this group of cases was appreciably less than that observed in the Atlanta patients. This was not due to the antigens, however, since comparative tests showed them to be slightly more sensitive than those employed in the first study. A possible factor in this connection is suggested by the fact that all of the patients had far advanced tuberculosis.

As controls, 13 additional serums were tested against the antigens. Of these, 7 were obtained from Battey Hospital patients and 6 from serums submitted for a routine Kahn test. One of them showed a slight reaction with the O antigen in a dilution of 1:80 and 2 caused agglutination in

the 1:40 dilution. No reactions were observed with the H antigen. The contrast between these results and those obtained with the serums of the patients is similar to that observed in the Atlanta study, though less pronounced.

DISCUSSION

In each episode the sequence of events and the evidence relating to the source of infection, incubation period and symptomatology followed the general pattern observed in outbreaks of food infection.

With reference to the bacteriological findings, certain observations suggest that the paracolon bacillus associated with these outbreaks was, in fact, the etiologic agent. It was recovered from the stools of 9 of the 12 patients, and no other pathogenic forms were recognized. In the Atlanta episode the stools of each patient yielded the organism in almost pure culture. At Battey Hospital the outbreak was limited to the 6 patients who were served a food mixture prepared less than 24 hours previously by an employee who was recovering from a diarrhea and whose stools were found to be positive. Moreover, the negative specimens from the 14 persons who were not ill indicate that the organism was not widely disseminated at the time of the outbreak.

The serologic findings also indicate a causal role on the part of the paracolon bacillus. In both outbreaks the serums from the patients agglutinated the antigens with greater frequency and in much higher titer than was observed among the controls.

SUMMARY

Two small outbreaks of acute gastroenteritis are described. A member of the Arizona group of paracolon bacteria was associated with both episodes. The epidemiologic, bacteriologic and serologic findings indicate that this organism was the etiologic agent.

THE RELATIVE STABILITY OF SCHICK AND DICK REACTIONS

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It has been the practice for many years to require that medical students assigned to clerkships in contagious diseases hospitals be Schick- and Dick-negative before reporting for duty. As a result of this policy, it is necessary to test each student for susceptibility to diphtheria and scarlet fever, and those found positive are subsequently immunized. This procedure is usually carried out by the Student Health Service. However, as a part of the course in Bacteriology, the students are Schick- and Dick-tested during an earlier period of their residence in school. It has become possible, as a result of this practice, to compare the Schick and Dick reactions in the same individuals at intervals of one to two years. During a period of 8 years, we have been able to gather data on 380 persons who have been tested twice with the indicated reagents.

Although there is a large literature attesting to the utility of Schick and Dick testing, it is surprising to find that there is little concerning the stability of these reactions in adult individuals who were neither immunized nor infected. Faulds[1] reported a study of nurses and found that of 149 nonreactors to the Dick test, all were still negative 3 to 6 months later. Peacock et al,[2] in the course of an extensive study of the relationship of the Dick test to scarlet fever, found that 16 of 78 nurses and internes who gave a previous history of being Dick-negative were positive on a repeat test. Schwentker et al[3] retested a large sample of a study group in Romania and reported that 5 to 8% of nonreactors later gave a reaction to scarlet fever toxin. There are, of course, other extensive studies[4–7] concerning the Dick test, but all are directed to evaluating the stability of the test with regard to immunization technics and the disease process.

Sellers et al[8] found that 3.7% of air force personnel who were nonreactors to the Schick test were positive when tested again 3 to 24 months later. Pulley and Fleisher[9] noted that 16.3% of medical students with a previous history of diphtheria, and therefore at one time presumably negative, reacted to the

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2. Peacock, S., Bigler, J. A. and Werner, M. 1939, Scarlet fever, hemolytic streptococci cultures and Dick tests in a children's hospital. Am. J. Dis. Child. **57:** 759–794.
3. Schwentker, F. E., Janney, J. H. and Gordon, J. E. 1943, The epidemiology of scarlet fever. Am. J. Hyg. **38:** 27–98.
4. Toomey, J. A. 1941, Scarlet fever immunization. Ann. Int. Med. **15:** 959–973.
5. Smiley, H. E. 1934, Observations relating to the specificity of the Dick test. J. Lab. & Clin. Med. **20:** 589–592.
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7. Little, G. M. 1945, Scarlet fever immunization in Edmonton, Alberta, Can. J. Pub. Health, **36:** 328–330.
8. Sellers, A. H., Baillie, J. H., Cruikshank, J. M. and McKibben, S. C. 1945, Findings on re-Schick testing air force personnel. Canad. J. Pub. Health, **36:** 390–395.
9. Pulley, H. C. and Fleisher, M. S. 1938, Schick reactions in students of medicine. Am. J. Pub. Health, **28:** 854–858, 1938.

Schick test. The same authors also reported that 35.1% of their study group who had been actively immunized against diphtheria at some previous time reacted to the Schick test. Others[10] have reported similar results with varying percentages.

MATERIALS AND METHODS

The subjects of this study were all medical students, primarily males, and almost exclusively from large urban environments. The average age was 22 years, although the range was from 18 to 38. Dick test toxin was purchased and was invariably from the same manufacturing source.* The test was carried out by a careful intradermal injection of 0.1 cc of material and was read at the end of 20 to 24 hours. An erythema of at least 10 mm in diameter was considered positive. The Schick test material was obtained from the State of Illinois Department of Health Laboratories and the test performed in a manner similar to that used for the Dick test. The reaction was read at the end of 48 to 72 hours and, in doubtful cases, was read again at the end of 5 to 6 days. No control tests were done with the Schick test and none were made of the original Dick tests. However, when individuals were re-Dick-tested after immunization, controls were performed with Dick toxin kept in a boiling water bath for one hour.

All the subjects of the study were Dick- and Schick-tested and then retested one to two years later. All those who reacted to the test material after the second test were immunized with the appropriate antigen regardless of the results of the first test. Dick toxin was used as the immunogenic agent for scarlet fever and was injected intracutaneously in five weekly doses of 0.1 cc, each containing increasing quantities of toxin as follows: 65 S.T.D., 250 S.T.D.. 1000 S.T.D., 3000 S.T.D. and 12,000 S.T.D. Diphtheria alum precipitated toxoid was used as the antigen for diphtheria immunization and was given in a single 0.1 cc intracutaneous injection which was repeated only if a re-Schick test was positive. Re-Dick and re-Schick tests were performed one month after the last immunizing injection.

TABLE 1.—*Stability of Dick tests.*

Class Number	Number of subjects	Initial reaction		Reversals			
				Positive→Negative		Negative→Positive	
		Positive	Negative	Number	Percent	Number	Percent
1	60	28	32	5	17.8	11	34.4
2	58	29	29	11	37.9	6	20.7
3	50	29	21	16	55.2	1	4.8
4	30	18	12	6	33.3	6	50.0
5	52	30	22	16	53.3	2	12.5
6	43	24	19	6	25.0	6	31.5
7	32	23	9	9	39.1	2	22.2
8	47	32	15	8	25.0	9	60.0
Totals	372	213	159	77	35.7	43	27.1

RESULTS

There were 380 persons included in the study, of whom 364 were tested with both Schick and Dick material. The remaining 16 individuals had either one or the other test. In table 1 are summarized the results of the Dick testing program. It is to be noted that on the initial test 213 subjects, or 57.3%, were positive. When they were retested one to two years later, 77, or 35.7%, did not react characteristically to the test material. Of the 159 subjects originally nonreactive, 43, or 27.1%, showed a reaction on retest. Combining the figures, we find that 120, or 32.2% of the 372 persons tested initially, reacted differently to the second test.

In table 2 are summarized the results of the Schick testing program. On the first test, 135, or 36.3%, of the subjects gave positive reactions. When they were retested, 33, or 24.4%, were Schick-negative. Of the 237 persons

10. Karelitz, S. and Maloshok, R. E. 1944, Immunity to diphtheria in army personnel. War. Med. **6**: 232–235.

* Parke, Davis & Co., Detroit, Mich.

TABLE 2.—*Stability of Schick tests.*

Class Number	Number of subjects	Initial reaction		Reversals			
		Positive	Negative	Positive→Negative		Negative→Positive	
				Number	Percent	Number	Percent
1	57	21	36	5	23.3	2	5.5
2	57	18	39	4	22.2	3	7.7
3	52	19	33	3	15.8	1	3.0
4	29	10	19	5	50.0	2	15.0
5	52	20	32	4	20.0	1	3.1
6	40	18	22	6	33.3	4	18.1
7	36	7	29	1	14.3	4	12.0
8	49	22	27	5	22.7	2	11.1
Totals	372	135	237	33	24.4	19	7.6

originally negative, 19, or 7.6%, were Schick-positive on retest. Combining these results to determine the total number of unstable reactions, we find that 52, or 14%, of the original 372 persons studied reacted differently to the second test.

As indicated in an earlier paragraph, all individuals positive to the second test were immunized with the appropriate antigen. Examination of the immunization records revealed that, of the 43 subjects changing from Dick-negative to Dick-positive (only 30 were re-Dick tested after immunization), 50% were still Dick-positive. Of the 15 positives after immunization, all but one reacted to the control material as well as the Dick test toxin. Of the 19 individuals changing from Schick negative to positive, 11 required one injection of the antigen and then were found to be Schick-negative. The remaining 8 subjects required two injections to change the reaction.

DISCUSSION

It appears from the evidence presented here and other reports in the literature that skin reactivity to standard doses of scarlet fever toxin or diphtheria toxin is not a fixed property. Phair[11] has shown, and it is widely recognized, that it is the antitoxin content of the body that determines whether or not a Schick test at any given time will be positive or negative. The antitoxin content is not constant and may fall below the critical level necessary to produce a negative skin test. If this be the case, and most evidence supports it, the demonstrated instability of the Schick and Dick reactions was not to be unexpected.

The purpose of this investigation was to compare the instabilities observed in the two tests, since they are both based on the same principle. As is indicated in the section on results, there were more than twice as many unstable reactions with the Dick tests than with the Schick tests. However, if the changes are examined separately, i.e., positive to negative change and negative to

11. Phair, J. J. 1942, Diphtheria immunization: the interpretation of the Schick test. Am. J. Hyg. **36**: 283–293.

TABLE 3.—*Comparison of the results of re-Schick and re-Dick testing one to two years after initial test.*

	Number of subjects	Positive→Negative		Negative→Positive		Combined changes	
		Number	Percent*	Number	Percent†	Number	Percent
Dick tests	372	77	35.7	43	27.1	120	32.2
Schick tests	372	33	24.4	19	7.6	52	14.0

* Percent of those initially positive.
† Percent of those initially negative.

positive change, we find that the former occurred with two-thirds the frequency among the Schick reactions as among the Dick reactions, but the latter occurred almost four times as frequently among Dick reactions as among Schick reactions.

The positive to negative change is one which may possibly be explained on the basis of external stimuli to antitoxin production, such as exposure to the etiologic agent or subclinical infection. This factor plus the normally expected fluctuation in antitoxin level would theoretically result in a negative reaction in a certain percentage of initially positive reactors. This type of change occurred in 35.7% of the Dick reactors and in 24.4% of the Schick reactors. Although there are more of these conversions among the Dick reactors, it is not felt that the difference is great enough to be significant. To determine whether this change was related to some characteristic peculiar to the individual, the records were examined to see whether a change in Schick reaction was accompanied by a change in Dick reaction in the same person. It was found that, of the 172 individuals showing changes of one kind or another in both tests, in only 10, or 3.5%, was there a simultaneous change of both reactions in the same subject. Apparently, this was no factor in the causation of the change.

The negative to positive change in reaction could only be dependent upon the fluctuations in antitoxin level and, therefore, was to be expected in a minimum of instances. This type of change occurred in 27.1% of the Dick-negatives but in only 7.6% of the Schick-negatives. If it may be assumed that the percent of changes of this type in Schick reactions are basal, then the occurrence of such a relatively large percentage of negative to positive changes among the Dick tests may throw some reasonable doubt on the true significance of a negative Dick test. The studies of Hooker and Follensby[12] indicate the presence of more than one erythrogenic toxin from streptococci of scarlatinal origin. This work lends further support to the possibility that dependence on the Dick test may not be as epidemiologically sound as dependence on the Schick test.

SUMMARY

A study of the relative stability of Schick and Dick reactions is described. Simultaneous tests were done in 364 of 380 adults. It was observed that positive to negative and negative to positive changes occurred. The former occurred at roughly the same rate in Schick reactors as in Dick reactors. The latter, however, occurred with almost four times the frequency among Dick-negatives as among Schick-negatives. The results are discussed in the light of the identity of the basic principle involved in both tests. The possibility is suggested that the Dick test may not be as epidemiologically sound as the Schick test.

12. Hooker, S. B. and Follensby, E. M. 1934, Studies of scarlet fever. II. Different toxins produced by hemolytic streptococci of scarletinal origin, J. Immunol. **27**: 177–193.

EXPERIMENTAL INFECTION OF THE UPPER RESPIRATORY TRACT OF YOUNG CHIMPANZEES WITH GROUP A HEMOLYTIC STREPTOCOCCI

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Much useful information about group A hemolytic streptococci and the infections caused by this group has been obtained by experimental work using small laboratory animals. The application of the results of such studies to problems arising from streptococcal infections in man has, however, been difficult. The reasons for this may lie in the artificial conditions required to produce infection in many of these animals and in the great differences between the two hosts.

Recently attempts have been made to overcome this difficulty. Watson et al[1] have reported a study of nasopharyngeal implantation of group A streptococci in rhesus monkeys. They showed that when group A streptococci became established and multiplied in the nasopharynx of these animals, a rise occurred in the antistreptolysin O titer of the serum. Furthermore, it was shown that type specific immunity developed following successful implantation of a strain. A peculiar feature of the disease in these animals, however, was that there was never any temperature elevation or other clinical manifestation of infection. Wilson et al[2,3] have studied experimental infections of the respiratory tract in rhesus monkeys with group C streptococci. These authors have emphasized, in particular, the added effect of infection with influenza A virus and of dietary and environmental factors on the course of these infections. Many of the results of these two studies may be applicable to the study of human infections with group A streptococci. However, information gained by studies of immunity to group C streptococcal infections cannot be applied to infections caused by members of group A. Furthermore, certain of the features reported by these authors appear to be peculiar to monkeys. The possibility arises that some other primate, in particular one of the anthropoid apes, might react to group A infections in a way more comparable to man.

Chimpanzees are the species of primate which would seem to most closely resemble man. They are susceptible to many human diseases. In particular, these animals have been infected with a variety of agents causing dis-

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* James Hudson Brown Memorial Fund Fellow, 1948–49.

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2. Wilson, H. E., Saslaw, S., Doan, C. A., Woolpert, O. C. and Schwab, J. L. 1947, Reactions of monkeys to experimental mixed influenza and streptococcus infections. An analysis of the relative roles of humoral and cellular immunity with the description of an intercurrent nephritic syndrome. J. Exper. Med. **85:** 199–215.
3. Saslaw, S., Wilson, H. E., Doan, C. A., Woolpert, O. C. and Schwab, J. L. 1946, Reaction of monkeys to experimentally induced streptococcus hemolyticus, group C, infection. An analysis of the relative roles of humoral and cellular immunity under conditions of optimal or deficient nutrition. J. Exper. Med. **84:** 263–276.

eases of the human upper respiratory tract.[4–6] There have been two reports of early attempts to transmit scarlet fever to these animals as a means of isolating the etiological agent.[7,8] Most of these experiments were complicated by the introduction of a variety of material by other routes, but in two there was fever and some evidence of pharyngeal infection after exudate from a patient with scarlet fever was introduced into the animal's throat. It appears that infection probably was successful in these animals, although bacteriological and immunological methods for proving this were not available at that time. It seemed worthwhile to attempt to induce experimental infections of the upper respiratory tract in this species with group A streptococci, and if such infections could be induced, to study the clinical course, immunity and possible sequelae. The present report is concerned with the results of such a study. In particular, the attempt was made in this study to observe animals which were subjected to repeated attempts to infect with heterologous types of group A streptococci.

4. Dochez, A. R., Shibley, G. S. and Mills, K. D. 1930, Studies on the common cold. IV. Experimental transmission of the common cold to anthropoid apes and human beings by means of a filterable agent. J. Exper. Med. **52:** 701–716.
5. Long, P. H., Bliss, E. A. and Carpenter, H. M. 1931, Etiology of influenza: transmission experiments in chimpanzees with filtered material derived from human influenza. J.A.M.A. **97:** 1122–1127.
6. Rich, A. R., Long, P. H., Brown, J. H., Bliss E. A. and Holt, L. E. 1936, The experimental production of whooping cough in chimpanzees. Bull. Johns Hopkins Hosp. **58:** 286–306.
7. Grunbaum, A. S. 1904, Some experiments on enterica, scarlet fever, and measles in the chimpanzee. Brit. Med. J. **II:** 816–819.
8. Landsteiner, K. and Levaditi, C. 1911, Essais de transmission de la scarlative aux singes. Ann. Inst. Pasteur, **25:** 754–775.

MATERIALS AND METHODS

Three healthy young chimpanzees ranging in weight from 13 to 16 pounds at the start of the experiments and estimated to have been between 1½ and 2½ years old at that time were used. Two were females (Becky and Donna) and one a male (Alamo). During the experiments the animals were kept in one room in large, individual wire cages about 8 to 10 feet apart. They were observed daily for any signs of illness. Daily rectal temperatures were taken when possible, and the temperature was considered to be significantly elevated if it reached 101 F. Gowns and masks were worn for protection of personnel, but no attempt was made to isolate the animals from each other. The various procedures were carried out without anesthesia. The experiments covered a period of 220 days.

Strains of streptococci.—One strain of each of 5 different types of group A hemolytic streptococci was used. These strains were 35RS75 (type 18), IRP43 (type 19), IRP41 (type 28), Griffin (type 12) and Kay (type 3). Three of these (35RS75, IRP43 and IRP41) had been isolated from human throats during respiratory infections occurring in association with an attack of rheumatic fever, and had been stored by lyophilization.* The other 2 strains (Griffin and Kay) were isolated from uncomplicated cases of streptococcal pharyngitis and used after a short period of storage in blood broth cultures at 4 C. During the course of the experiments all 3 animals were inoculated with each of these 5 strains. In addition, strain 35RS75a, derived from strain 35RS75 after a period of multiplication in the pharynx of one of the animals (Alamo), was inoculated into the throat of Donna 11 weeks after she had been successfully infected with strain 35RS75. The inoculation procedure in each experiment consisted of the instillation into the animal's throat of 0.5 cc of a 5- to 7-hour-old blood broth culture of the type being tested.

At various times during the course of the experiment the attempt was made to infect these animals with viruses in the hope that if the viral infection could be established, an enhancement of the streptococcal infection might result. There were three instances where these attempts were followed by febrile episodes suggesting that a viral infection had occurred, but no findings characteristic of the diseases appeared. In only one of these three was there any suggestion that the

* These three strains were kindly furnished by Dr. Rebecca C. Lancefield, the Rockefeller Institute for Medical Research, 66th St. and York Ave., New York City.

clinical course was altered in any way. This will be commented on later.

Throat cultures.—Cultures were obtained from the animals' throats at frequent intervals starting one week before the first experiment. During the period immediately after inoculation, cultures were obtained at weekly, and in many instances, shorter intervals. These throat cultures were made on rabbit blood agar plates and incubated at 37 C for 18 to 24 hours. When β hemolytic streptococci were present an attempt was made to identify them according to serological group and type by the capillary pipette techniques of Lancefield[9] and Swift, Wilson and Lancefield.[10]

Tests for antibodies.—Blood was obtained at frequent intervals from the femoral or anticubital veins. One cc of this blood was usually oxalated and used for determination of the sedimentation rate in Wintrobe tubes. The remainder was allowed to clot, and the serum was removed and stored at −20 C. The titers of antistreptolysin O and antistreptokinase and the γ globulin levels were determined on each serum specimen. Tests were also made for the presence of type specific bacteriostatic antibodies. The method for determining the antistreptokinase titer was essentially the same as that described by Anderson et all.[11] It was found that satisfactory results were obtained when the quantities of serum and reagents used by these authors were halved. This was done in order to conserve material. The serum dilutions recommended by Kaplan[12] were used in this test. The antistreptolysin O titers were determined by the method of Hodge and Swift.[13] The dilutions of serum used in this test differed by an increment of 50 instead of the system described by these authors. Determination of the fraction of serum proteins precipitated by zinc sulfate* was done simultaneously on all of the serums of each animal by the method described by Kunkel,[14] using the modification of the standard introduced by Melnick.[15] This fraction is referred to as γ globulin in the text and tables and is expressed in turbidity units. Tests for type specific bacteriostatic antibodies were done by the indirect method on serum specimens selected from those obtained before and after the inoculation of each type of streptococcus. This test has been described in detail by Rothbard.[16] This test was carried out with undiluted serum only. The antibacterial test was considered positive if there was a ++ difference in growth from at least two dilutions of organisms when compared with the preinoculation serum and the saline controls. In both of the other antibody tests a rise in titer of two tubes or more has been found to be a significant change and was therefore considered to be a positive result. In the test for γ globulin a difference in turbidity of 1½ units or more represents a significant difference when the tests on a group of unknown serums on one animal are run simultaneously.

Criteria of experimental infection.—The essential event which constitutes infection with the hemolytic streptococcus would appear to be the invasion of the host's tissues by the streptococcus. In this study the results of three different antibody tests, together with the throat cultures and "clinical" observations, particularly the animals'

9. Lancefield, R. C. 1928, The antigenic complex of streptococcus hemolyticus. I. Demonstration of a type specific substance in extracts of streptococcus hemolyticus. J. Exper. Med. **42:** 91–104.
10. Swift, H. F., Wilson, A. T. and Lancefield, R. C. 1943, Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exper. Med. **78:** 127–134.
11. Anderson, H. D., Kunkel, H. G. and McCarty, M. 1948, Quantitative antistreptokinase studies in patients infected with group A hemolytic streptococci: A comparison with serum antistreptolysin and gamma globulin levels with special reference to the occurrence of rheumatic fever. J. Clin. Investigation, **27:** 425–434.
12. Kaplan, M. H. 1946, Studies of streptococcal fibrinolysin. III. A quantitative method for the estimation of serum antifibrinolysin. J. Clin. Investigation, **25:** 347–351.
13. Hodge, B. E. and Swift, H. F. 1933, Varying hemolytic and constant combining capacity of streptolysin; influence on testing for antistreptolysins. J. Exper. Med. **58:** 277–288.

* The γ globulin determinations were kindly made by Miss Nada Ledinko.

14. Kunkel, H. D. 1947, Estimation of alterations of serum gamma globulin by a turbidometric technique. Proc. Soc. Exper. Biol. & Med. **66:** 217.
15. Melnick, J. L. and Ledinko, N. Unpublished data.
16. Rothbard, S. J. 1945, Bacteriostatic effect of human sera on group A streptococci. I. Type-specific antibodies in sera of patients convalescing from group A streptococcal pharyngitis. J. Exper. Med. **82:** 93–106.

rectal temperatures, have been considered in attempting to determine whether the above criterion had been satisfied. Of these, an increase in one or more of the antibody titers would appear to be the most reliable indication that actual tissue invasion had occurred, for without actual invasion it is not likely that bacterial antigens would be present in the host in sufficient quantities to serve as a stimulus to antibody production. The failure of antibodies to appear would seem to indicate that if infection did occur, it was so localized as a result of natural or acquired immunity that the absorption of bacterial products was negligible. A persistently positive throat culture not accompanied or followed by a rise in antibody titer might indicate then that there was either an extremely localized superficial area of invasion, or simple colonization on the surface. These two cannot be distinguished in the present series of

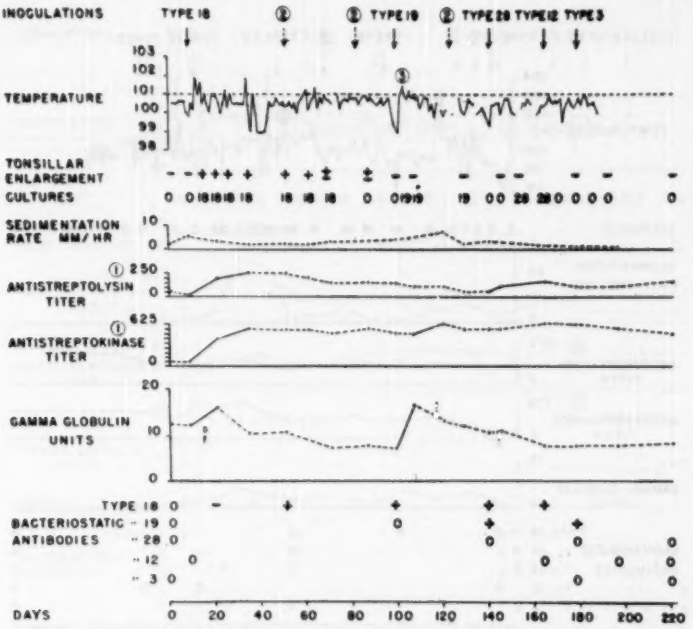

FIG. 1.—Experimental results, Alamo.

The results of throat cultures (designated CULTURES in the figure) are indicated by 0 if no group A streptococci were found, and by a number indicating the type if group A streptococci were present.

Figure "1" encircled: Each subdivision of the scale represents one dilution in the series used in that particular test. Periods during which a significant rise in titer occurred have been indicated by connecting the points with a solid line.

Figure "2" encircled: The broken lines with arrow indicate the times during the course of the study when this animal was given other "infectious" agents.

Figure "3" encircled: During this period this animal developed a nasal discharge, sneezing and a slight cough, and fever which lasted for three days. All three of the animals had some fever at about this time.

experiments. A negative throat culture only indicates that the organisms were not present at the particular site touched by the swab. It would obviously be possible for an area of infection to be present in a less accessible part of the nasopharynx. In accordance with this reasoning, it has been assumed that the occurrence of a significant rise in titer in one or more of the antibody tests used is acceptable evidence that tissue invasion has taken place, even though the inoculated organism cannot be recovered from the throat. The other findings are of value primarily in determining the extent of this invasion.

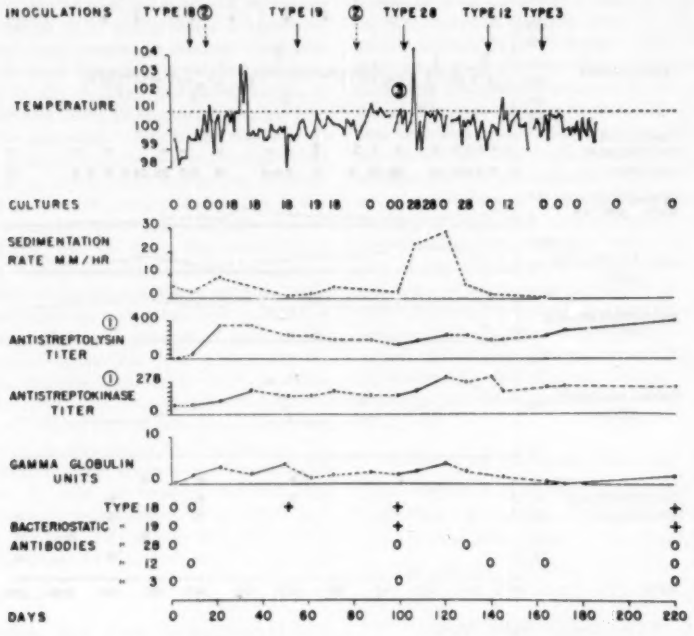

FIG. 2.—Experimental results, Becky.
Legend as in figure 1.

EXPERIMENTAL RESULTS

The results of the experiments are shown in figures 1, 2, and 3 and summarized in table 1. A total of 16 inoculations were carried out. One of these consisted of the reinoculation of an animal with a strain of streptococcus which had been successfully established in the animal's throat in a previous experiment. Of the remaining 15 experimental inoculations, 10 were followed by a significant rise in titer in at least one of the antibody tests used, indicating that an appreciable amount of tissue invasion had occurred. The throat culture remained positive for at least one week in 8 of these. In one experiment there occurred a persistently positive throat culture for one week even though no rise occurred in any of the antibody titers. In 5 experiments, including the one in which a strain was introduced into an animal's throat for

the second time, none of these findings was present.

Fever and clinical signs.—In each of 7 experiments there was an elevation of the rectal temperature to 101 F or more starting within a few days of inoculation. In 2 of the experiments there was an alternative possible cause for the fever, and fever, therefore, can definitely only be said to have occurred in 5 experiments. One of these 2 animals (fig. 2, type 18) was inoculated with throat washings and serum from an individual having an illness diagnosed clinically as epidemic pleurodynia 7 days after the streptococcal inoculation and 2 days before the first day of fever. In this case, the throat cultures, which had been negative, became positive during the period of fever. In the other instance where the fever that followed inoculation might possibly have been related to some other cause, the animal (fig. 1, type 19) had a febrile period lasting 4 days, and displayed the clinical features of the common cold starting 2 days after the date of inoculation. Since the other two animals also had non-streptococcal upper respiratory infections at this time and had temperature elevations lasting one or two days along with these findings, it is quite possible that the fever in this animal was also due to this cause.

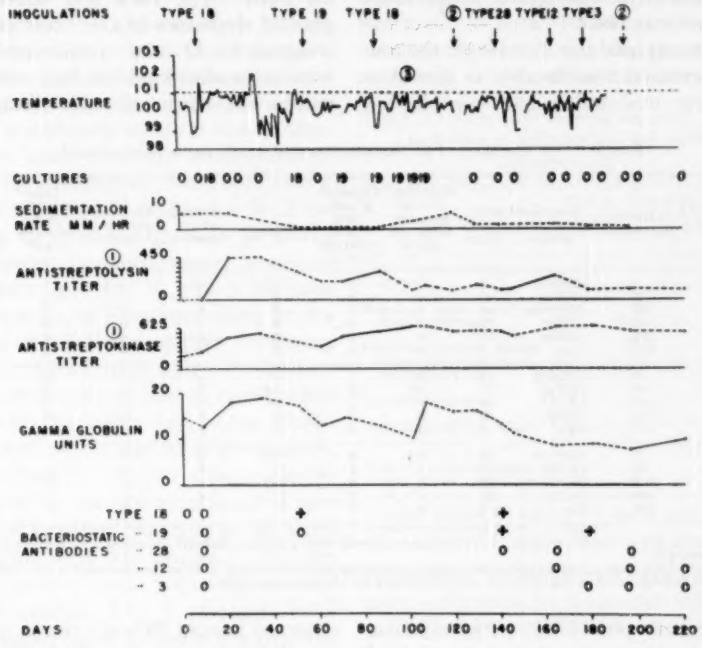

FIG. 3.—Experimental results, Donna.
Legend as in figure 1.

The incubation period before the onset of the temperature elevation averaged 4.6 days in the remaining 5 cases, and the duration of the febrile period averaged 5 days. The incubation period and the duration of the fever

were quite variable, however, the former varying from 1 to 8 days and the latter from 1 to 11 days. The longer febrile periods were usually intermittent. The elevation was usually not very high, reaching 102 F on only 5 of a total of 29 febrile days. The highest temperature reached was 104.4 F.

In one animal (fig. 1, type 18) the tonsils increased considerably in size after the first inoculation and contained a number of pits filled with exudate. This change persisted for a period of about two months, after which they decreased in size and appeared somewhat cleaner. During the period of tonsillar enlargement, culture of the throat consistently yielded heavy growths of the inoculated type of organism. This animal had the longest febrile period of any, extending intermittently over the first half of the period of tonsillar enlargement. Except for this one instance, the changes in the throats were not impressive. Usually there was no noticeable change in behavior or other evidence that the animal was not well. In one instance, however, there was a considerable degree of prostration evident in association with a temperature jump to 104.4 F. As far as could be determined the animals were in as good health at the end of the experimental period as they were at the beginning.

Changes in antibody titers and γ globulin levels.—The rises and subsequent gradual decreases in the titers of antistreptolysin O and antistreptokinase were somewhat variable, but were well within the range of such changes as observed in man. When a rise in titer in one of these tests occurred following the introduction of a new strain of streptococcus, a significant increase in titer was always reached by the third week after inoculation, except in one experiment in which the animal was not bled at that time (fig. 2, type 3). The test for type specific antibacterial antibodies is not a quantitative test and can therefore be considered only in terms of positive or negative. It was uniformly negative for a particular type in serum obtained from the animal before that type was inoculated into the animal's pharynx, and in 6 instances became positive afterwards. There were no instances in which this test became negative during the course

TABLE 1.—*A summary of the inoculations and results in the three animals.*

Animal	Day of experiment	Inoculated with		Duration of temperature elevation (days)	Duration of positive throat cultures (weeks)	γ globulin*	Antistreptolysin test*	Antistreptokinase test*	Type specific antibacterial tests*	Total number of positive findings
		Strain	Type							
Donna	9	35RS75	18	11	6	+	+	+	+	6
	56	1RP43	19	0	6	+	+	+	+	5
	87	35RS75a	18	0	0	0	0	0	0	0
	140	1RP41	28	0	0	0	+	0	0	1
	164	Griffin	12	0	0	0	0	0	0	0
	178	Kay	3	0	0	0	0	0	0	0
Alamo	9	35RS75	18	9	9	+	+	+	+	6
	99	1RP43	19	4†	4	+†	0	+	+	4
	140	1RP41	28	0	3	0	+	0	0	2
	164	Griffin	12	0	0	0	0	0	0	0
	178	Kay	3	0	0	0	0	0	0	0
Becky	9	35RS75	18	6†	8	+†	+	+	+	4
	54	1RP43	19	1	1	0	0	0	+	3
	103	1RP41	28	4	4	+†	+	+	0	4
	140	Griffin	12	1	1	0	0	0	0	2
	164	Kay	3	0	0	?	+	0	0	1

* The definition of a positive result (+) in the three antibody tests and the γ globulin determinations is given in the section on methods.

† These findings have a questionable relationship to the streptococcal infection. They are not included in the total number of positive findings listed on the right, nor were these data used in preparing table 2.

of the experiments after a positive result was once obtained. In table 2 it can be seen that there was a positive result in 1 or more antibody tests in 10 experiments, 2 or more in 6 experiments, and in all 3 tests in 4 experiments.

The levels of γ globulin show considerable variation between the 3 animals. In spite of this, the levels are fairly constant in individual animals. In each of the 6 instances where a rise in titer in 2 or more antibody tests occurred following inoculation, there was also a rise in the level of γ globulin. In 3 instances there was a question as to the relationship between these, however. In Becky (see fig. 2) the γ globulin level appears to have been rising at the time of the first inoculation. Following the nonstreptococcal upper respiratory infection which occurred in each animal on about the 100th day of the study, there was also a rise in γ globulin. In Donna (see fig. 3, [3]) this is clearly unrelated to the introduction of a new strain of streptococcus, but in the other 2 animals it is not apparent whether one or both of these factors are related to the charge. The γ globulin levels also tended to drop back towards the preinoculation value during the weeks after the peak level.

Recovery of the organism from the throat.—In 9 of the experiments the type of streptococcus inoculated into the animal's throat was cultured from the throat one week or longer after the inoculation date. The cultures remained positive for a period of time varying from one week, which was usually the earliest culture attempted, to 9 weeks. In only 2 instances did the culture remain positive for as short a period as one week. In the experiments in which the organism was recovered from the throat at any time after inoculation it could be isolated from there for an average of 4.6 weeks.

Sedimentation rates.—The erythrocyte sedimentation rate was used primarily as a means of indicating the possible occurrence of any sequelae of these infections. For this reason no attempt was made to obtain bleedings for sedimentation rates during the period immediately after inoculation in each experiment although this was done in some instances. At times the sedimentation rate was elevated when determined soon after inoculation. In general, little can be said except that even when elevated during the immediate postinoculation period, it had usually returned to the preinoculation rate with three weeks, when it was checked at this time. There was no evidence revealed by the use of this test that any of the sequelae of streptococcal infections, as encountered in man, occurred in these animals.

TABLE 2.—*Correlation between the results of the antibody tests and other findings in the 16 experiments.*

	Number of antibody tests positive			
	0	1	2	3
Total number of experiments	6	4	2	4
Number with positive throat cultures	1	2	2	4
Number of weeks during which throat culture remained positive	0, 0, 1 0, 0, 0	0, 0 1, 3	4 4	6, 6 8, 9
Number with fever	1	1	1	2*
Number of days of fever	0, 0, 0 0, 0, 1	0, 0 0, 1	0, 4	0, 0 9, 4
Number showing significant rise in γ globulin	0	0	0	3*

* Only changes for which there was no other possible cause are included here. See text.

Correlations between the presence of various positive findings.—In table 2 the data are arranged to show the extent of correlation between the length of time the organism was able to maintain itself in the throat, the occurrence of and

duration of fever, and the extent to which the animals responded with the production of various antibodies and γ globulin. It is apparent that a correlation does exist. In the 4 experiments in which a positive result was obtained in all 3 of the antibody tests, the throat culture was positive for at least 6 weeks in all 4; and 2 animals had fever for at least 9 days. Of the 4 having only 1 positive antibody test, 1 had a positive throat culture for 1 week, 1 for 3 weeks, and 1 had fever for 1 day.

TABLE 3.—*The effect of multiple infections with heterologous types on the production of type specific antibodies.*

Order of inoculation attempt in each animal	Data abstracted from report of Watson, Rothbard and Swift		Results of this study		Totals	
	Number of successful implantations	Number followed by a rise in agglutination titer	Number of successful implantations	Number in which type specific antibodies appeared	Total number of experiments	Total number of positive results
1	5	5	3	3	8	8
2	4	4	3	3	7	7
3	3	1	3	0	6	1
4	1	0	0	0	1	0
5	0	0	1	0	1	0

This is a composite table constructed from data from these experiments and data abstracted from Watson, Rothbard and Swift.[1]

Attempts to cause repeated infections in each animal.—Implantation of one type of streptococcus in an animal's throat did not prevent other types from becoming established at a later date. In 2 of the animals 3 successive types apparently became implanted, while in the third animal the evidence indicates that at least 4 and possibly all 5 strains successfully established themselves. In only one experiment was a particular type reintroduced into an animal's throat after a previous successful implantation of that type. This attempt did not succeed, although the animal subsequently proved to be susceptible to several other types. It is significant in this regard that type specific antibodies were demonstrable following more than half of the inoculation attempts that were accompanied by some other evidence that implantation had occurred.

A column in table 1 summarizes the number of positive findings following each inoculation. In general, the number of positive findings was less and less with each successive experiment. Each successive infection was, then, progressively less extensive than the preceding. Although the 5 strains of streptococcus used in these experiments were inoculated into each of the 3 animals in the same order, it is unlikely that each strain used was progressively less virulent than the preceding one in the exact order of the experiments. It seems more probable that the animals were becoming more resistant. The possibility that there is some degree of heterologous immunity following repeated group A streptococcus infections is supported by the data reported by Watson et al[1] in their experiments in monkeys. The interpretation of their data in the way it is used here is based on the evidence presented above that there is a correlation between the severity of a particular infection and the number of antibodies that an animal will produce in response to it. In the work reported here, type specific antibacterial antibodies appeared after the first two inoculations in each animal, but not after subsequent ones. Watson et al do not present a

detailed report of their results with the test for antibacterial antibodies. They do, however present the results of type specific agglutinin tests.

The results of the type specific antibody tests in the 2 groups of experiments are compared in table 3. It can be seen that such antibodies appeared after the first 2 successful inoculations in all of the animals in both series of experiments. In one of their animals they appeared after the third implantation. In the 7 remaining instances in their work and that reported here, where inoculation of a 3rd, 4th, or 5th type of streptococcus was followed by other evidence of successful implantation, no type specific antibodies appeared.

DISCUSSION

It seems fair to say that experimental "acute follicular tonsillitis" was produced in at least one of the animals by inoculation with a strain of group A streptococcus. In this experiment there occurred fever, tonsillar enlargement and exudate starting 2 days after inoculation with a strain of type 18 streptococcus. The throat culture became positive for this type of organism, and there was a significant rise in titer of antistreptokinase and antistreptolysin O. Type specific antibacterial antibodies were demonstrable in serum obtained after infection, but not in that obtained before. In 3 other experiments there occurred fever which was not attributable to any other cause, positive throat cultures for at least one week and a rise in titer in at least one antibody test. Although the findings of acute follicular tonsillitis were not seen in the throats of these animals, the continued presence of the inoculated organism is good evidence that the infection which caused the animal's fever and rise in antibody titer was located somewhere in the nasopharynx. In 6 of the experiments fever was not observed, or could not be definitely attributed to the streptococcal infection, although a rising titer of one or more antibody tests indicated that tissue invasion had occurred. Four of these animals had positive throat cultures at least one week after inoculation. The results in these 6 experiments resemble those reported by Watson et al[1] in monkeys. It has been pointed out by these authors that such findings may be compared to instances of human subclinical infections.

In these experiments the long continued presence of the inoculated organism in the throat was associated with an increase in titer of 2 or 3 of the antibody tests used, while when this period was short it was usual for one or none of the antibody tests to give a positive result. This was not a simple cause and effect relationship, however, since the rise in antibody titer had always reached a maximum by about the third week after inoculation and the organism usually continued to be present for 3 to 5 weeks after this time. Fever, although not present in all of the experiments, tended to occur more frequently and for longer preriods in the animals that showed a positive result in 2 or 3 of the antibody tests used. This suggests the logical conclusion that the extent and severity of the early part of the infection bore a significant relationship to the amount of penetration of bacterial antigens into the host and the ability of the organism to maintain itself in the pharyngeal tissues. Actually, the infections which would be judged more severe by these criteria were the first few in each of the animals. With each successive inoculation attempt there tended to be fewer positive findings, suggesting that group A streptococcal infections may result in some increase in resistance to infection with

heterologous types in addition to homologous type specific immunity. One of the tests which failed to give a positive result after the earliest experiments was that for determining type specific antibacterial antibodies. A similar tendency has been pointed out in the data of Watson et al.

SUMMARY AND CONCLUSIONS

1. The results of the experimental inoculation of young chimpanzees with group A streptococci have been reported. In this work it was conclusively shown that these animals are susceptible to pharyngeal infection with these organisms and that clinical findings similar to those that occur in man may appear.

2. The results were compatible with the theory that immunity to streptococcal infection is chiefly type specific. Data were presented suggesting that in addition there may be a gradual increase in resistance to infection with heterologous types following repeated infection with a series of strains of group A streptococci.

3. No sequelae were observed to occur in these animals.

REPRODUCTION-INHIBITING AND PARASITICIDAL EFFECTS ON *PLASMODIUM GALLINACEUM* AND *PLASMODIUM LOPHURAE* DURING INITIAL INFECTION AND HOMOLOGOUS SUPERINFECTION IN CHICKENS

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Earlier, we (1945) studied the immological relationships of *Plasmodium gallinaceum* and *Plasmodium lophurae* by testing homologous and heterologous immunity to superinfection of sporozoites and erythrocytic stages in chickens previously infected with each species. The present work is limited to a study of initial infection and homologous superinfection by erythrocytic stages with special reference to merozoite production and survival and death rates of the parasites formed, as has been done by us for initial infections of *Plasmodium brasilianum* in Central American monkeys (1944), *Plasmodium cynomolgi* (1947) and *Plasmodium knowlesi* (1949a) in rhesus monkeys, and by Thompson (1944) for *Plasmodium floridense* in lizards. These studies followed from work on *Plasmodium cathemerium* by Boyd and Allen (1934) and Boyd (1939) on initial infection and by Boyd and Gilkerson (1942) on superinfection.

Our results are of particular interest in that they give direct evidence that much of the lowering of the number of merozoites produced by segmenters, which reaches its maximum near the crisis, is due to acquired immunity. This study carries further an analysis previously made on reproduction-inhibiting and parasiticidal effects during the course of a few initial infections with these two avian species incident upon our studies of the antimalarial effects of a nitrogen mustard (1948) and of quinine (1949b).

The work on immunity to superinfection has been recently reviewed by W. H. Taliaferro (1949), but Boyd and Gilkerson (1942) are the only investigators who have studied rates of reproduction during superinfections as far as we are aware. They found that *P. cathemerium* injected into canaries latently infected with the homologous species produced 75 to 78% as many merozoites after 8 to 10 hours as were produced in canaries initially infected.

MATERIALS AND METHODS

The same strain of *P. gallinaceum* and of *P. lophurae* and the same procedures were used in this and the previous study by us (1945). At the beginning of the present work, each strain of parasite had been maintained for a year or more continuously in chickens by the intravenous injection of parasitized blood once or twice a week. All initial and superinfections were blood-induced. The superinfecting dose varied from 1 to 25 ml of heavily parasitized citrated blood per 100 g chicken or approximately 2 to 50×10^9 parasites. It was considered more advisable to indicate the superinfecting dose by a parasite count from a blood smear made 3 to 5 minutes after the injection (see column 5 in tables 1 and 3) rather than by the number of parasites injected per weight of chicken. All parasite counts are given as a number per 10,000 red blood cells.

Our methods of differentiating reproduction-inhibiting and parasiticidal effects of immunity (1) by determining the primary rate of reproduction from the merozoite mean in conjunction with the length of asexual cycle and (2) by comparing the number of merozoites formed with the actual increases and decreases in blood population of the parasites have been fully described in earlier papers by us (1944, 1947 and 1949a).

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To ascertain the length and synchronism of the asexual cycle, differential counts were made of parasites every 4 to 10 hours. These consisted of classifying a random sample of 50 to 100 parasites into 4 groups of (1) very small ones, (2) parasites intermediate in size with one nucleus, (3) parasites with 2 through 4 nuclei, and (4) parasites with 5 or more nuclei. Gametocytes were also classified but are disregarded in this study because they seldom formed more than 1% of any population. To represent the asexual cycle the percentage of multinucleated parasites (groups 3 and 4) and of parasites with 5 or more nuclei (group 4) are graphed in figure 7. In this figure maximal segmentation takes place just after each peak in multinucleated stages.

A merozoite mean per segmenter for a given interval was obtained after tabulating the number of merozoites in each of 25 or more mature segmenters from ordinary thin dried blood films stained with Giemsa. Maturity of a segmenter was considered to have been reached when some of the divided nuclei had separated into merozoites. To insist that all of the merozoites be mature might have made the counting of merozoites in some segmenters easier, but would not have avoided all errors because the merozoites often clumped together while drying. Individual merozoites of segmenters with 4 to 12 merozoites possessed more cytoplasm and less contracted nuclei than segmenters with more merozoites. They were, however, often indistinguishable from immature multinucleated forms. When segmenters were scarce, they were located with a 4 mm dry objective (×45) on a blood film evenly spread with a thin layer of oil, and their merozoites were counted with an oil immersion objective (×90). Segmenters could be found at all hours because of the somewhat asynchronous asexual cycle, but were most numerous within a given 36-hour cycle for 12 hours after the peak in multinucleated forms and were most mature during the last 4 of the 12 hours. Technical difficulties in counting merozoites in segmenters, especially when bunched, made accurate merozoite means per segmenter impossible. For example, we were often uncertain whether a given segmenter contained 21 or 22 merozoites and were sometimes unable to place the number between 19 and 22 merozoites. Nevertheless, the merozoite means per segmenter are sufficiently accurate for the conclusions based upon them in this paper.

Changes in the basic rate of reproduction were considered to take place when the length of the asexual cycle or the mean number of merozoites per segmenter changed. The fate of free merozoites at a given segmentation was approximated as shown in the following example. When 24 merozoites were produced (merozoite mean per segmenter) during a given 36 hours, and the parasitemia increased from 5 to 20 (factor of 4) and decreased to 10 during the same period, we may conclude that 4 parasites (free merozoite survival rate) of the 24 produced invaded new red cells but that only 2 managed to survive the 36-hour period (over-all survival rate), or, conversely, that 20 failed to invade new red cells and 2 died during intracorpuscular growth. As pointed out in a recent paper by us (1949b), however, determinations of the parasiticidal effects of immunity against *P. gallinaceum* are invalidated to a certain extent by any influx of exoerythrocytic stages, and the somewhat asynchronous asexual cycle in both species gives a falsely low figure for the survival of merozoites during segmentation because any nonsegmenting fraction of a population decreases during intracorpuscular growth as the segmenting population increases. In experiments reported in this paper, the influx of exoerythrocytic stages was minimized by studying blood-induced infections, and the number of merozoites which entered or failed to enter red cells are only approximations.

The same scale was used for the data graphed in the figures. Similarly, the same type of line was used for the parasite counts, merozoite means per segmenter, etc. (see explanation in fig. 2). Initial infections are represented by thin solid lines, superinfections by heavy lines.

EXPERIMENTAL RESULTS

1. Initial infections with P. gallinaceum.—A schematic representation of the somewhat asynchronous cycle of *P. gallinaceum* in chickens is shown in figure 1. Under the 12-hour light and dark routine maintained in our animal quarters, the peak in multinucleated forms during the acute rise of initial infections occurred at 36-hour intervals around midnight or noon and the peak of mature segmenters occurred about 8 hours later. The periodicity is accentuated in this figure because the various percentages could not be accurately represented by drawing only three parasites at every time interval. Typically, the asexual cycle takes 36 hours, as reported by Giovannola (1938), Lumsden and Bertram (1940) and Taliaferro, Coulston and Silverman (1944), but is often

delayed or breaks up into two or three minor broods as described by W. H. and L. G. Taliaferro (1949b). Segmenters of any one asexual cycle divided into a variable number of merozoites from 4 to 40, usually 10 to 36, with a merozoite mean throughout the course of infection varying in general from 14 to 26. The expected factor of increase in the parasitemia every 36 hours for this species, therefore, should be between 14 and 26. This was rarely the case. As shown in figure 2, it only occasionally—at the beginning of the infection—equalled the number of merozoites produced and decreased thereafter (cf. W. H. and L. G. Taliaferro, 1949b).

The first two parts of figure 2 show the average parasitemia curve, merozoite mean per segmenter, and over-all parasite survival and death rates every 36 hours during 18 initial infections. All chickens weighed approximately 500 g and were initially infected intravenously so that slightly more than 100 parasites were found in their blood immediately after. Ten of the chickens only lived 3 to 4 days (fig. 2, acute): the other eight lived 9 days or longer (fig. 2, semiacute). In both groups, the over-all survival rate of parasites for the first 36-hour segmentation (fig. 2, shaded area) was high. All of the 14.6 to 15.8 merozoites produced (fig. 2, merozoite mean) lived. During the second segmentation, the survival rate decreased to 4.6 and 6 in the two sets, respectively. During the third segmentation, it decreased to 1. Thus, of the 20 or more parasites produced per segmenter, only one survived and the rest (95% or more) died. Through this third segmentation, the parasites actually increased in number although at a decreasing rate and thereafter, in the semiacutely infected chickens, they just maintained themselves or actually decreased. This point, therefore, represents the *initial peak of the infection* in an immunological sense. It terminated the acute rise and initiated the parasite decline. As long as the parasites just maintained themselves, an average of one per segmenter per asexual cycle survived. When they decreased in number, less than one parasite survived.

No appreciable relapses occurred in these chickens. If they had, as they frequently do, parasite survival would have been proportionately reflected in the intensity of the relapse. In experiments in which relapses occurred, they tended to be less severe as the infection progressed. As far as we have tested, infection with *P. gallinaceum* may persist for 2½ years, but parasites were usually so scarce after a month or two that merozoite mean determinations could not be made.

These 18 infections were of further interest because the merozoite mean per segmenter was in general higher in the rapidly fatal infections than in the semiacute ones beginning at 12 hours after the injection of parasites. Merozoite means from samples of 25 to 30 segmenters from each of the 18 chickens at 6- to 12-hour intervals throughout the infections are shown in the third section of figure 2. Standard errors for these means varied between 0.6 and 1.1. Six determinations at similar times in the infections were made on the two sets before the acutely infected chickens died. Although two of these (the 3rd and 5th) were not or were barely significantly different ($P = 0.2$ and 0.05, respectively), the other four were statistically highly significant with $P < 0.0001$ (cf. the solid with the open triangles in the third part of fig. 2). These results indicate that a high merozoite mean per segmenter can in general be correlated with a high pathogenicity and, thus, conversely with a low immunity. Aside from this difference between acute and semiacute infections, these merozoite means are remarkable for their

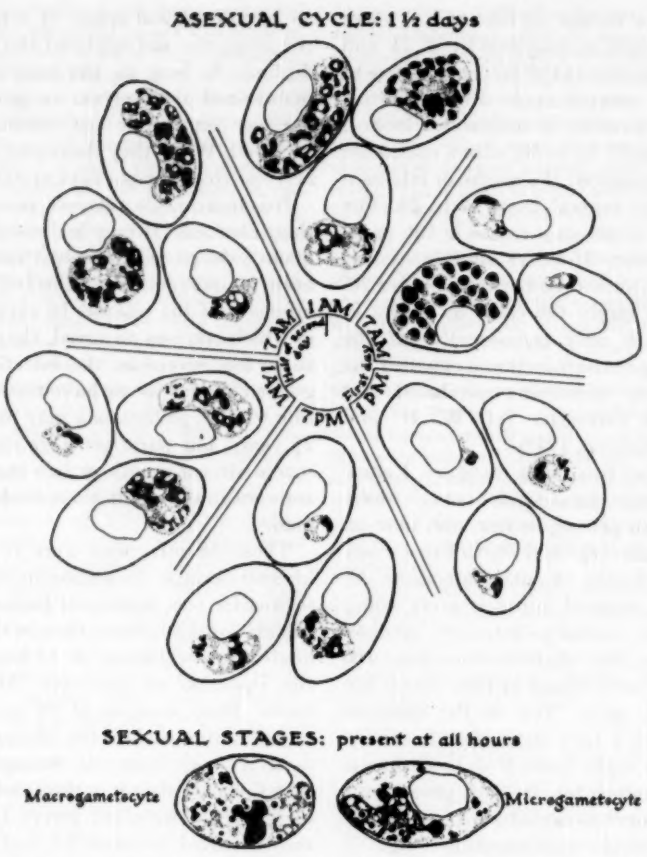

FIG. 1.—Representation of the 36-hour asexual cycle of *P. gallinaceum* (in chickens) constructed from camera lucida outlines of erythrocytic asexual stages and two gametocytes. ×2000. The cytoplasm of the parasite is lightly stippled, the nucleus is darkly stippled, and the pigment is indicated by black granules or masses.

Note that multinucleated forms were usually most numerous around midnight of one day and around noon 1½ days later.

wide range at any one time. For example, 50 hours after infection, in the midst of the acute rise, samples of 25 segmenters in the 18 chickens gave a range in merozoite mean between 17.4 ± 0.6 and 26.6 ± 0.6. Furthermore, the merozoite mean of 26.6 consisted of segmenters containing 18 to 36 merozoites, whereas the mean of 17.4 consisted of segmenters containing 12 to 24 merozoites. Such disparities are invariably encountered and represent to us inherent differences in individual chickens.

In spite of variability in chickens, both with respect to their capacity to acquire immunity and their innate suitability as a medium for the reproduction of the parasites, there was a definite over-all trend in the production of parasites, as shown by the merozoite mean. It was usually high during the acute rise of the infection; then, if the

chicken did not die, it definitely declined, even though the parasitemia maintained itself, and thereafter proceeded in an irregular wave-like manner during the developed infection (fig. 2, merozoite mean of semiacute infection). These decreases amounted roughly to 15 to 34%. When the merozoite mean was low, segmenters often appeared dark and contracted. Such forms were designated crisis forms by W. H. and L. G. Taliaferro (1934). They have been seen in simian, saurian and other avian malarias by Afridi (1938) and by us (1934, 1944, 1947, 1949a and b) and Thompson (1944). The asexual cycle was also sometimes delayed in part or as a whole to the extent that reproduction could be considered to have been inhibited an additional 2 to 6%, as already reported by us (1949b).

From these data it may be concluded that during the acute rise of the infection, the initial high rate of reproduction of *P. gallinaceum* decreased 20 to 40%, and parasites were destroyed at a rap-

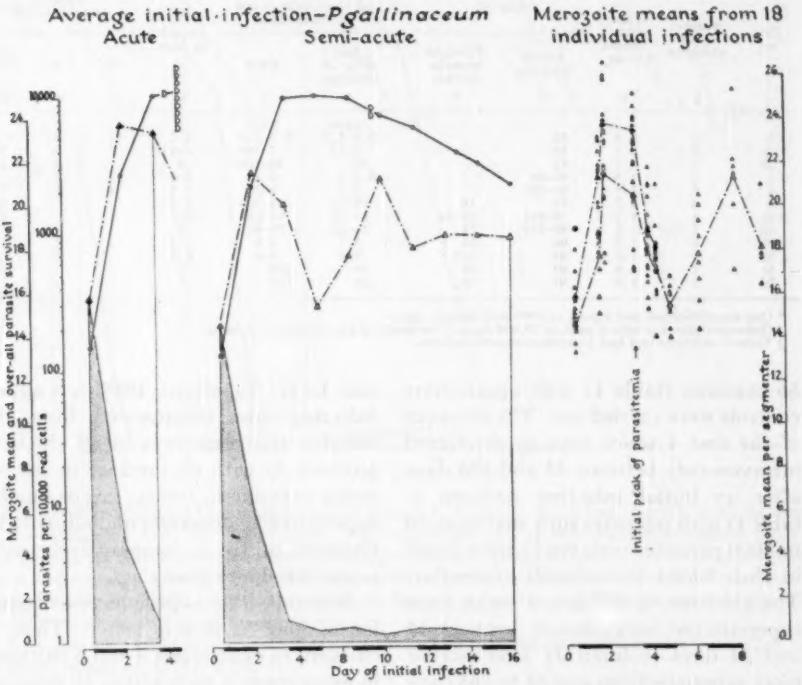

FIG. 2.—Blood-induced initial infection of *P. gallinaceum* in chickens. The parasitemia, rate of reproduction (merozoite mean) and over-all parasite survival and death rates are shown by averages from 10 acute and 8 semiacute infections. Individual merozoite means are given for all 18 (solid triangles, acute; open triangles, semiacute) infections in the third section of the figure.

Merozoite mean, dot and dash line; parasitemia, solid line; over-all parasite survival per segmenter, shaded area; over-all parasite death per segmenter, clear area under merozoite mean; D, dead; 36-hour periods, light and heavy segments of the base line.

Note that the rate of reproduction is high throughout although it varies within a range of 33% (16 to 24 merozoites per segmenter) and tends to decrease after the peak in numbers, while the initial high survival rate rapidly decreases.

idly increasing rate. Thereafter, the rate of reproduction fluctuated somewhat but almost all of the parasites were destroyed unless a relapse occurred. These changes were largely dependent upon the development of acquired immunity by the chicken as is shown later.

2. Superinfections with P. gallinaceum *and control initial infections.*—Seven series of superinfections involving 56 chickens (table 1) with appropriate controls were carried out. The chickens of the first 4 series were superinfected intravenously between 45 and 186 days after an initial infection (column 3, table 1) with parasites such that from 30 to 4000 parasites (column 5) were found in their blood immediately thereafter. The chickens of the last 3 series were superinfected intravenously between 18 and 34 days (column 4) after one or more superinfections and 52 to 183 days after an initial infection (column 3) with parasites such that from 300 to 4000 parasites (column 5) were found in their blood immediately thereafter. These experiments were designed to test whether immunity to superinfection was stronger when the initial peak was high, whether it persisted 186 days after an initial infection, whether it was reinforced by one or more superinfections and what mechanisms were involved.

In experiment 1, the parasitemia during the initial infection was differentially suppressed by giving quinine (peaks of 140 to 10,000). Forty-five days later when parasites were scarce and quinine had disappeared (cf. W. H. Taliaferro and Kelsey, 1948, and W. H. and L. G. Taliaferro, 1949b), a superinfecting dose disappeared from the blood within two days in all chickens (column 7) with no increase in parasitemia (column 6). Hence, this or smaller superinfecting doses can undoubtedly be disposed of by a comparatively subacute initial infection.

Immunity to superinfection lasted for as long as it was tested. Thus, 5 chickens in experiment 3 and 6 chickens in experiment 4 were tested 50 through 186 days after their initial infection with a superinfecting dose similar to that in experiment 1. Parasites progressively decreased in all chickens until they could not be found after 2 to 4 days (column 7). The extra 2 days sometimes required to clear the blood of parasites in these experiments as compared to

TABLE 1.—*Seven experiments containing 4 to 14 chickens homologously superinfected with* P. gallinaceum. *Chickens in experiments 5 through 7 were superinfected prior to the test superinfection.*

Exp. no.	Number of test chickens	Approximate weight in kg	Day of: Initial infection	Day of: Previous superinfection	Parasite count per 10,000 red blood cells of test superinfection: Just after i.v. injection	Parasite count per 10,000 red blood cells of test superinfection: Peak	Length in days	Type of superinfection: A	Type of superinfection: B	Type of superinfection: C
	1	2	3	4	5	6	7	8	9	10
					×10³	×10³				
1	14	0.4	45		0.5–2.5	0.5–2.5	½–2	11	3	
2a	8	0.7	56		7–12	7–19*	2–3½*	2	5	1
2b	2	0.7	52		40	50–160	4		1	1
3	5	1.5	50–84		0.3–4	1–4	2–4	2	3	
4	6	1.7	97–186		2–4	2–4	2–3	4	1	1
5	5	0.7	52	18	15–24	34–200	3–6†			4†
6a	4	1.3	73–183	23–34	16–19	17–150	2½–7			4
6b	2	1.5	90–174	23	26–40	26–40	4			2
7a	6	1.3	80	28	5–7	5–7	2–4		2	1
7b	2	1.3	80	28‡	3–4	3–4	2–3		2	
7c	1	1.8	80	28	20	120	7			1
7d	1	1.3	80	28‡	20	120	7			1

* One superinfection had a peak of 3000 and lasted 7 days.
† One superinfection with a peak of 18,000 caused the death of the chicken in 3 days.
‡ These 3 chickens had had 4 previous superinfections.

experiment 1 may have been due to differences in size of the chickens or to chance individual variations instead of a lowering of titer. Immunity to superinfection probably lasts much longer—at least as long as the initial infection lasts (see p. 277).

chickens of experiment 7a, which was slightly smaller than that given to the 8 chickens in experiment 2a, persisted for about the same number of days (column 6). Extremely large doses of parasites when given as a 2nd or 5th superinfection after 28 days (experiment 7c and d)

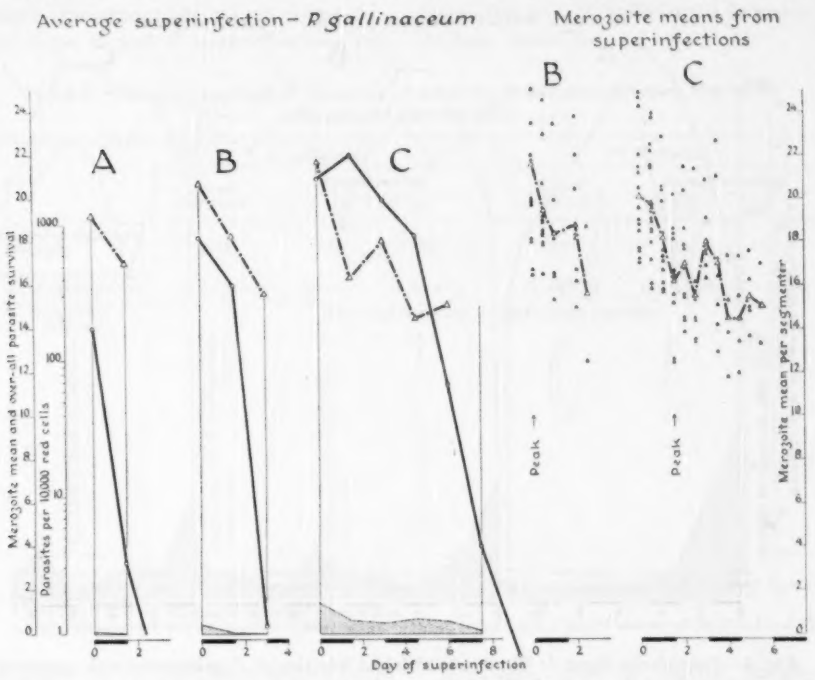

FIG 3.—Blood-induced homologous superinfection (A, B and C depending roughly upon the size of the challenging dose) of *P. gallinaceum*. The parasitemia, rate of reproduction (merozoite mean) and over-all parasite survival and death rates are shown by averages from 18, 13 and 11 superinfections, respectively. Individual merozoite means (solid triangles) are given for 6 type B and 11 type C superinfections in the 4th and 5th sections of the figure. Counts at 2- or 3-hour intervals are aligned. For other explanations, see legend in figure 2.

Note that the rate of reproduction decreases sooner and that the survival rate is never as high as in initial infections (cf. fig. 2 and 4).

Immunity seemed not to be markedly increased by a previous superinfection with the doses and intervals of time used in experiments 5 through 7. The previous superinfections reached peaks of over 2000 in experiment 5 and of 200 to 300 in the rest. The test superinfection (column 5, table 1) in the first 6

may have been less efficiently disposed of (column 6) than after an initial infection of 52 days (experiment 2b).

Over-all parasite survival and death rates every 36 hours and merozoite means per segmenter were obtained for all superinfections as they were for initial infections (fig. 2). The 56 superin-

fections could be divided into types A, B and C (columns 8 to 10, table 1) largely depending upon the density of the parasitemia immediately after superinfection. Average values are shown in the first three sections of figure 3. The over-all survival was less than half a segmentation. Parasites were too scarce in type A to make any more determinations. In type C, it decreased to a low point of 14.5 (i.e., a 32% reduction) at the 4th segmentation. A low merozoite mean was usually associated with the occurrence of varying numbers of crisis

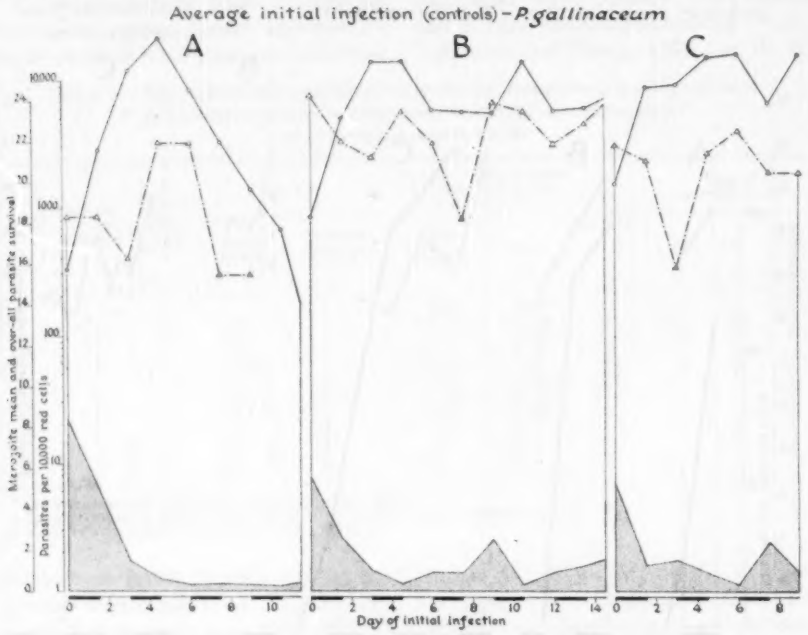

FIG. 4.—Controls for figure 3: Blood-induced initial infection of *P. gallinaceum* with inoculums comparable to those given type A, B and C superinfections. The parasitemia, rate of reproduction (merozoite mean) and over-all parasite survival and death rates are shown by averages from 7, 6 and 3 infections, respectively.

For other explanations, see legend in figure 2.

parasite per segmenter for types A and B and was 0.5 or less for type C except for the first segmentation (1.2) and during a slight relapse (0.7) at the 4th segmentation. These values represented a minimal death rate of 95% and usual death rates of 97% or more.

Average merozoite means per segmenter of the inoculated material varied from 19 to 21.4 in the 56 superinfections (type A, B, and C, fig. 3). It decreased 10% or more in all of them at the second forms. These findings definitely associate the lowering of the merozoite mean of the segmenters with the parasiticidal action of acquired immunity and add evidence in support of the authors' contention (1944, 1947, and 1949a) that a decrease in the merozoite mean is in part due to acquired immunity. As just described, the increased death of parasites and occurrence of crisis forms, normally associated with the crisis in initial infections, begins almost immedi-

ately and progresses more rapidly in disappearing superinfections. Similarly, the merozoite mean begins to decrease much earlier and often reaches a lower value faster in superinfections than in initial infections.

Merozoite means per segmenters made throughout the course of individual type B and C superinfections are referred to during initial infections. In addition, there was a 9 to 11% difference in the merozoite mean per segmenter of initial and superinfections as indicated from counts of the merozoites of all segmenters examined during the course of this study in initial infections and in superinfections of sufficiently immune chickens (table 2).

TABLE 2.—*Percentage frequency of the number of merozoites produced in segmenters from initial infections and superinfections.*

	P. gallinaceum			*P. lophurae*		
	Initial infection†	Superinfection§		Initial‡ infection	Superinfection‡§	
		A & B	C		A & B	C
Number of segmenters examined	7765	874	1964	2415	539	1512
Average merozoite mean per segmenter	19.7±0.06	18±0.2	17.5±0.1	11.4±0.04	10.5±0.04	11.1±0.05
Number* of merzoites	Percentage frequency of merozoites per segmenter					
6	0.5	<0.1	<0.1	2	4	1.5
8	0.5	1	1	12	21	13.5
10	1.8	4	5	27	32	29.8
12	5.5	12	11	39	31	38
14	10	14	17	16	10	14
16	14	16	18	3.5	1.8	3
18	15	14.5	15	0.5	0.2	0.2
20	14	12	11.3			
22	12	9	8			
24	11	9	6			
26	7	4	3			
28	4.5	2.5	3			
30	2.5	1.5	1			
32	1	0.5	0.6			
34	0.5	<0.1	<0.1			
36	0.2	<0.1	<0.1			

* Segmenters with odd numbers of merozoites from 5 to 35 are included in the next larger even number class.
† During the first 6 days 0.1% of the segmenters contained 38 or 40 merozoites and thereafter 0.1% contained 4 merozoites.
‡ Less than 1% of the segmenters contained 4 merozoites.
§ More parasites were injected into type C superinfection than into type A and B superinfections (cf. fig. 3, 5, 8 and 10)

graphed on the right hand side of figure 3. They are oriented with regard to the peak in parasitemia of the superinfection. The peak took place during the first 36 hours in type B. In type C superinfections, it usually occurred at the end of 36 to 48 hours but in two of them occurred 72 hours after the test dose. These last two superinfections closely simulated initial infections and represented an overpowering of the existing immunity. The individual merozoite means (right hand part of fig. 3) varied over a wide range for any given interval of time during the course of the superinfections in various chickens. This kind of variation has been previously

Averages from 12 control initially infected chickens, i.e., of the same weight and given the same dose of parasites as were the immune chickens, are given for every 36 hours in figure 4. Two of the four chickens recovered in control group A, one of four recovered in control group B, and all died in control group C. The general trends in these control infections were similar to those described earlier (cf. fig. 2). The lower over-all survival at the first segmentation in the infections shown in figure 4, as compared to the 100% survival shown in figure 2, slight differences in the parasitemia curves and the generally smaller merozoite means represent

variations often encountered during initial infections. Both sets of controls, however, differed from the superinfections in their more intense parasitemia, higher survival rate and larger merozoite mean (table 2) which usually de-

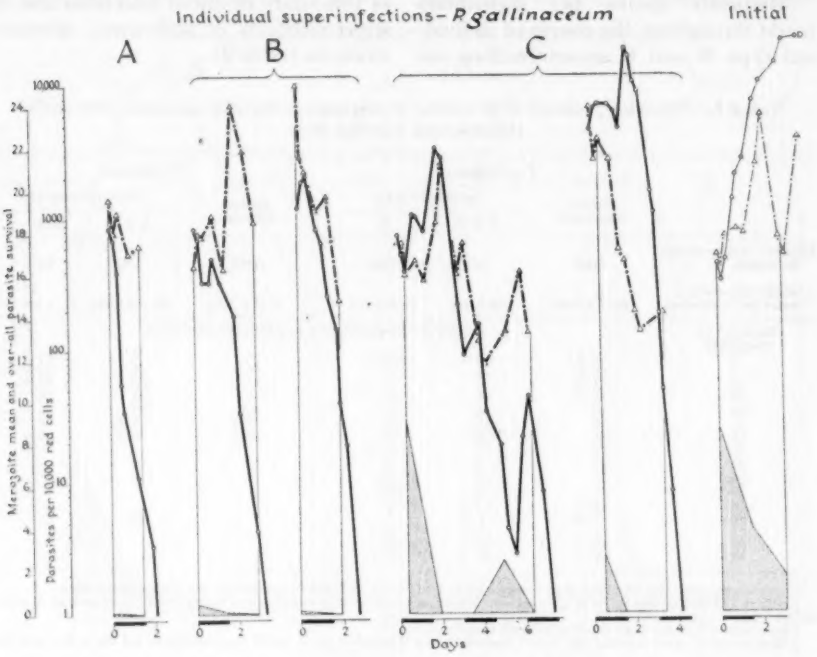

FIG. 5.—Homologous type A, B and C superinfections of *P. gallinaceum* from 5 chickens and of one control initial infection receiving the type B amount of inoculum.

For other explanations, see legend in figure 2.

Note that the parasitemia curves in type B and C superinfections increase periodically due to free merozoite survival at segmentation. The increases are not commensurate with the actual number produced.

creased more slowly (cf. fig. 3 and 4). It was only after the initial peak of the infection that they resembled the superinfections to a certain extent. These differences are evident by comparing figures 2 and 4 with figure 3 and the data in table 2.

As has already been mentioned, the parasitemia steadily decreased at overall 36-hour intervals except at the beginning of type C superinfections. On the other hand, the parasitemia often temporarily increased within an asexual cycle at the time of segmentation in type B and C superinfections due to the survival of free merozoites. In type B, such increases varied between 1 and 1.5 times and in type C between 2 and 4 times. These increases were remarkable in view of the fact that parasites were disappearing so fast and in view of the further fact that rate increases of free merozoites during the first 2 or 3 segmentations in the controls only ranged between 2 and 6. Parenthetically, it may be mentioned that these comparatively low values in initial infections were probably correlated with the large size of the chickens.

Determinations every 6 to 12 hours

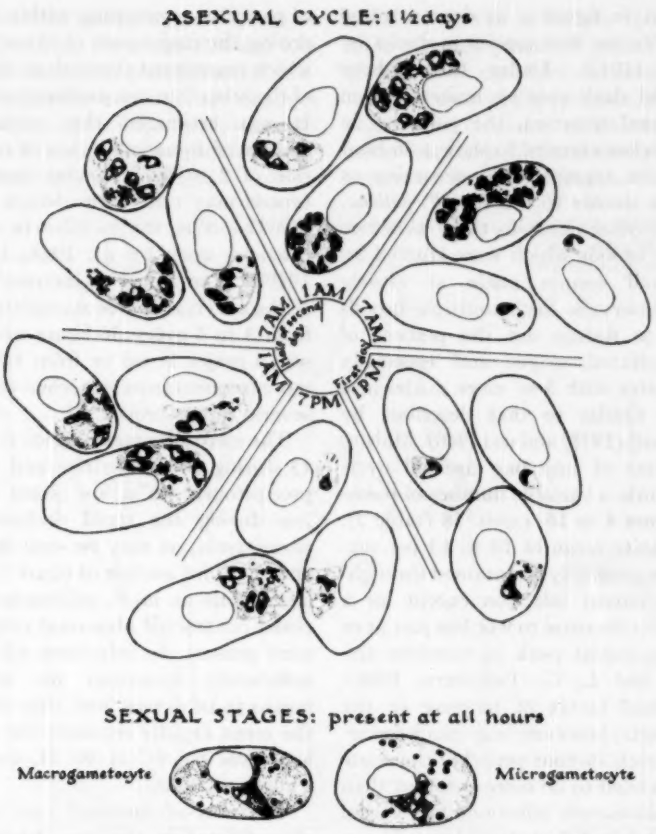

FIG. 6.—Representation of the 36-hour asexual cycle of *P. lophurae* (in chickens) constructed from camera lucida outlines of erythrocytic asexual stages and two gametocytes. ×2000.
For other explanations, see legend in figure 1.

from typical infections of each of the three types of superinfections are graphed in figure 5 along with one control initial infection. They illustrate the periodic increases due to the survival of free merozoites. Thus, in the 4th superinfection in figure 5, there was an increase in parasitemia from 350 to 1200 (3.4 times) 12 hours after injection, another from 770 to 3000 (4 times) after 1½ days, and a third from 90 to 140 (1.5 times) after 3½ days. An unusual relapse occurred in this chicken on the 6th day of its superinfection with a marked increase to two parasites in survival rate. Segmentation occurred after 24 hours when the inoculum consisted largely of intermediate sized uninucleated parasites (first type B and C in fig. 5) and immediately when it contained a large number of segmenters (second type B and C in fig. 5). The chicken with the second type C superinfection in figure 5 recovered in spite of the large number of parasites inoculated. It had considerable acquired immunity.

Initial infections with P. lophurae.—A schematic representation of the asexual cycle of *P. lophurae* in chickens

is shown in figure 6 as demonstrated previously for this species in ducks by Hewitt (1942). Under the 12-hour light and dark routine maintained in our animal quarters, the parasites in some chickens tended to show a 36-hour cycle with segmentation occurring at intervals similar to those of *P. gallinaceum*. In other chickens there were two or three broods which were studied by differential counts made at closely spaced intervals. Such multiple broods tended to flatten out the percent of multinucleated stages and especially of parasites with 5 or more nuclei in a manner similar to that described by Coggeshall (1938) and us (1940). Mature segmenters of any one asexual cycle divided into a variable number of merozoites from 4 to 16, rarely 18 (table 2). A merozoite mean of 10 to 13 per segmenter was usually maintained throughout the patent infection except for a temporary decrease to 9 or less just at or after the initial peak in numbers (cf. W. H. and L. G. Taliaferro, 1948). The actual factor of increase in the parasitemia, however, was much lower. During each 36-hour period of the acute rise, it tended to be more constant than in *P. gallinaceum* infections but varied from 2 to 6 in different chickens. It was <1 during the crisis.

This strain of *P. lophurae* was nonfatal to chickens except when large numbers of parasites were given. Therefore, infections of *P. lophurae* were characterized by an acute rise, a peak in numbers, a parasite decline and a latent infection (cf. W. H. and L. G. Taliaferro 1945 and 1948 and Reilly et al, 1949). The type of infection is illustrated by data from two chickens in figure 7. One infection (L1) had one clearly defined brood which segmented every 36 hours and was delayed 18 to 24 hours as it passed through the crisis and approached a low grade (6th to 7th day). The other infection (L2) had two broods of parasites segmenting within 12 hours during the major part of the acute rise, which rearranged themselves at the end of the crisis into one predominant brood. It was estimated that such delays amounted to an inhibition of reproduction of 2 to 6%. Varying numbers of broods may arise from delays incident upon handling the parasites in vitro (cf. previous work by us, 1928, 1934 and 1949b). The factor of increase because of the survival of free merozoites varied from 3 to 4 every 36 hours when there was a major brood or from 1.5 to 2 at more frequent intervals when there were several minor broods.

The merozoite mean varied from 10 to 13 during the acute rise and declined precipitously to a low point of 9 or less during the rapid decline of the parasitemia, as may be seen in L1, L2 and the third section of figure 7. At this latter time as in *P. gallinaceum* infections, contracted abnormal crisis forms were present. In infections which were sufficiently numerous for merozoite means to be determined after the crisis, the mean rapidly regained the previous level (see fig. 6C of W. H. and L. G. Taliaferro, 1948).

The over-all survival rate of parasites differed in the two chickens, but tended in each chicken to be constant during the acute rise and to decrease at the initial peak of the infection and thereafter (fig. 7).

Relapses were infrequent, but when they occurred the merozoite mean approached the value found during the acute rise and the over-all survival rate rose to one or more. The infection lasted at least $1\frac{1}{2}$ years, i.e., as far as it was tested.

From these data, it may be concluded that the rate of reproduction of the parasites was maintained at a fairly constant rate except for a temporary inhibition of about 20 to 30% at the crisis, whereas only one quarter to one half of

the merozoites produced per segmenter survived during the acute rise and less than one merozoite per segmenter survived during the crisis. These changes were largely associated with the development of acquired immunity by the chicken as shown later.

after an initial infection (column 3, table 3) with parasites such that from 100 to 3000 parasites (column 5) were found in their blood immediately thereafter. The chickens of the last three series were superinfected intravenously between 6 and 19 days (column 4) after

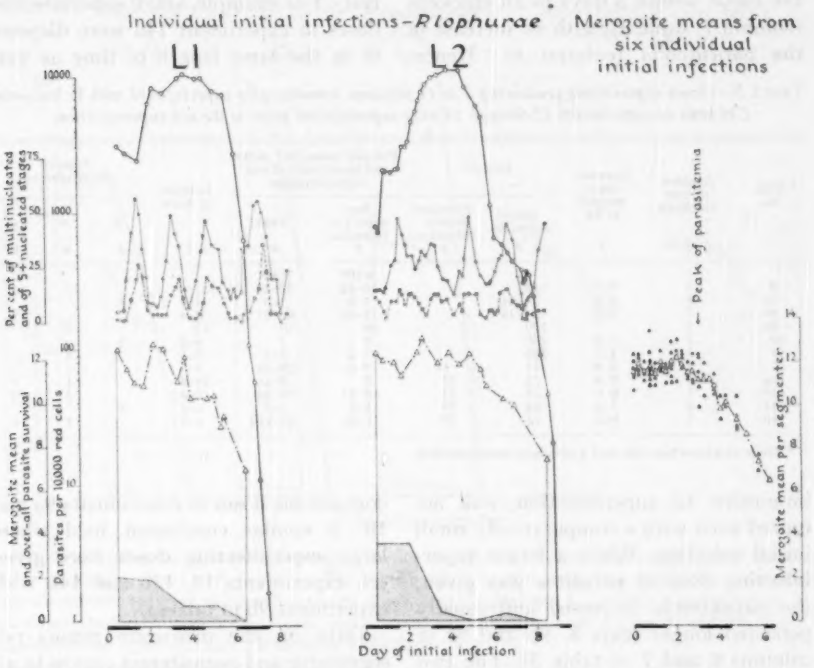
Individual initial infections – P. lophurae
Merozoite means from six individual initial infections
L1
L2
Per cent of multinucleated and of 5+ nucleated stages
Merozoite mean and over-all parasite survival
Parasites per 10,000 red cells
← Peak of parasitemia
Merozoite mean per segmenter
Day of initial infection

FIG. 7.—Blood-induced initial infection of *P. lophurae*. The parasitemia, rate of reproduction (merozoite mean and length of the asexual cycle), and over-all parasite survival and death rates are shown from two chickens. Individual merozoite means are given from 6 infections in the 3rd section of the figure.

Percent multinucleated stages, dotted line with circles; percent parasites with 5 or more nuclei, dotted line with squares. For other explanations, see legend in figure 2.

Note that the rate of reproduction is moderate as compared to *P. gallinaceum* (11 to 13 merozoites per segmenter) during the acute rise and tends to decrease 33% or more after the peak in numbers, while the survival rate is never high, but tends to be more or less constant during the acute rise and decrease after the peak in numbers.

4. Superinfections with P. lophurae *and control initial infections.*—Seven series of experiments involving 39 chickens (table 3) with appropriate controls were carried out. The chickens of the first 4 series were superinfected intravenously between 15 and 259 days

one or more superinfections and 25 to 44 days after an initial infection (column 3) with parasites such that 100 to 2300 parasites (column 5) were found in their blood immediately thereafter. The effectiveness of an initial infection and of one or two superinfections was thus

tested under varying conditions. Survival and death rates were ascertained throughout.

Peaks in the initial infection in the 8 chickens of experiment 9a ranged from 100 to 10,000. Eighteen to 64 days later, a superinfecting dose was cleared from the blood within 3 days in all chickens (column 7, table 3), with no increase in the parasitemia (column 6). Hence, immunity to superinfection was acquired even with a comparatively small initial infection. When a larger superinfecting dose of parasites was given, the parasitemia increased and usually persisted longer (exp. 8, 9b and 9c in columns 6 and 7 of table 3). The two chickens in experiment 8 possibly took longer than the rest of the chickens to dispose of their superinfection because they had been severely pecked by larger chickens several days before the test superinfection.

Immunity to superinfection lasted for as long as it was tested although it had decreased somewhat in titer 259 days after the initial infection. Thus, the parasitemia of the superinfecting dose of parasites increased (cf. columns 5 and 6) and the blood infection lasted longer (column 7) in all 5 chickens of experiment 11 as compared to those of experiment 9a.

A superinfection prior to the test superinfection seemed not to fortify markedly the initial infection within the particular range of doses and time intervals used. (The previous superinfections reached peaks of 2400 in experiment 12 and approximately 300 in the rest). For example, small superinfecting doses in experiment 14a were disposed of in the same length of time as were comparable doses in experiments 9a and 10. A similar conclusion held where large superinfecting doses were given (cf. experiments 12, 13b and 14b with experiment 9b in table 3).

Data on the merozoite means per segmenter and parasitemia curves in all the *P. lophurae* superinfections were treated as were those of *P. gallinaceum* and could conveniently be divided into three types depending upon the amount of inoculum (fig. 8). A superinfecting dose producing a parasitemia of about 220 (type A, fig. 8) with a merozoite mean per segmenter of 10.2 for the inoculated segmenters disappeared so rapidly from the blood that only a few segmenters were found during the subsequent 36 hours. These gave a mean of 8. The over-all survival rate for this 36-hour period averaged one fifth of a merozoite. A larger superinfecting dose such that the parasite count was 400 to

TABLE 3.—*Seven experiments containing 2 to 11 chickens homologously superinfected with* P. lophurae. *Chickens in experiments 12 through 14 were superinfected prior to the test superinfection.*

Exp. no.	Number of test chickens	Approximate weight in kg	Day of:		Parasite count per 10,000 red blood cells of test superinfection		Length in days	Type of superinfection		
			Initial infection	Previous superinfection	Just after i.v. injection	Peak		A	B	C
	1	2	3	4	5	6	7	8	9	10
					$\times 10^2$	$\times 10^2$				
8	2	0.1	15		16–22	22–48	3½–6		1	1
9a	8	0.9	18–64		1–3	1–3	1–3	8		
9b	2	0.5	23–24		15–30	25–67	3–4			2
9c	1		100		10	10	2½		1	
10	2	1.6	160		2	2	1–3	2		
11	5	1.7	259		1–2	3–5	2–4		5	
12	2	0.4	31	6*	9–12	20–44	3–5		1	1
13a	2	0.6	25	13	5	5–13	2½–4		1	1
13b	5	0.6	25	13	15–17	15–34	2–3½		3	2
14a	3	1.8	44	19	1–3	1–3	1–3	3		
14b	7	0.6	13	19	17–23	47–110	4–5		1	6

* These chickens had also had a previous superinfection.

2300 in the test chicken also progressively decreased (type B, figure 8) but persisted longer and showed a slightly higher survival rate for the first 36 hours. Average merozoite means for the same intervals were 11.3 and 10.3, respectively. The parasitemia of all type C 0.002, respectively. At 36 hours or more crisis forms were prevalent.

Merozoite means per segmenter made through individual type B and C superinfections are graphed on the right hand side of figure 8. They tended to decrease more rapidly in type B superinfections

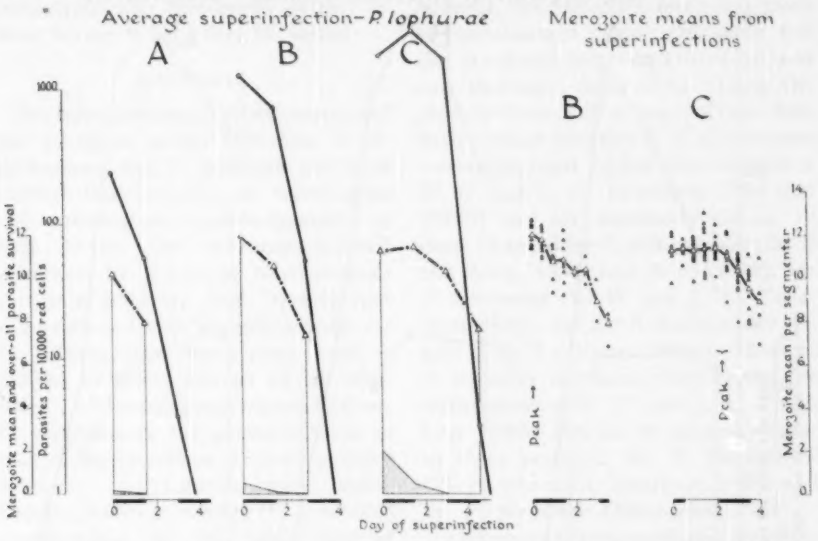

FIG. 8.—Blood-induced homologous superinfection (A, B and C depending roughly upon the size of the challenging dose) of *P. lophurae*. The parasitemia, rate of reproduction (merozoite mean) and over-all parasite survival and death rates are shown by averages from 8, 8 and 12 superinfections, respectively. Individual merozoite means (solid triangles) are given from 8 type B and 12 type C superinfections in the 4th and 5th sections of the figure.

For other explanations see legend in figure 2.

Note that the rate of reproduction decreases sooner and that the survival rate is usually lower and decreases faster than in initial infections (cf. fig. 7 and 9).

superinfections increased before decreasing. The dose for this type with a resulting parasite count of 900 to 3000 in the test chicken overlapped the dose for type B—probably due to variability in the amount of immunity possessed by individual chickens. As may be seen in type C, figure 8, the average merozoite mean remained the same for two segmentations and then decreased to a low point of 7.7, while the average over-all survival rates for the three corresponding asexual cycles were 2.0, 0.5, and

than in type C superinfections and the merozoite mean especially from type A and B superinfections was smaller than that from all initial infections (table 2).

The main divergences of superinfections (fig. 8), provided the test doses were not too large, from control initial infections of *P. lophurae* (fig. 7 and 9) were: (1) the over-all parasitemia progressively decreased, (2) the merozoite mean decreased sooner and more precipitously, and (3) fewer parasites sur-

vived, or reciprocally, more died. Only one of the 15 controls died.

The survival of free merozoites at segmentation could not be accurately studied in this species because of asynchronism and the presence of multiple broods. Nevertheless, when frequent parasite counts were made (fig. 10), type A was characterized by no survival of over one merozoite per segmenter (producing no temporary increases in parasitemia), type B by infrequent survivals of over one, and type C by invariable increases from 1 to 2 (occasionally to 3.5) merozoites at two segmentations.

Timing of the increases was related to the number of broods of parasites in a given population. For example, there were obviously two broods of parasites in the second type C superinfection in figure 10. There was an increase in parasitemia from 1740 to 3900 (2.2 times) immediately after superinfection, from 4000 to 4800 (1.2 times) at 12 hours, from 4800 to 5400 (1.1 times) at 36 hours, and from 6100 to 11,000 (1.8 times) at 48 hours. The stage of parasite injected and amount of synchronism in a given population determined the time and extent of the segmentation increases as with *P. gallinaceum*. The frequency of

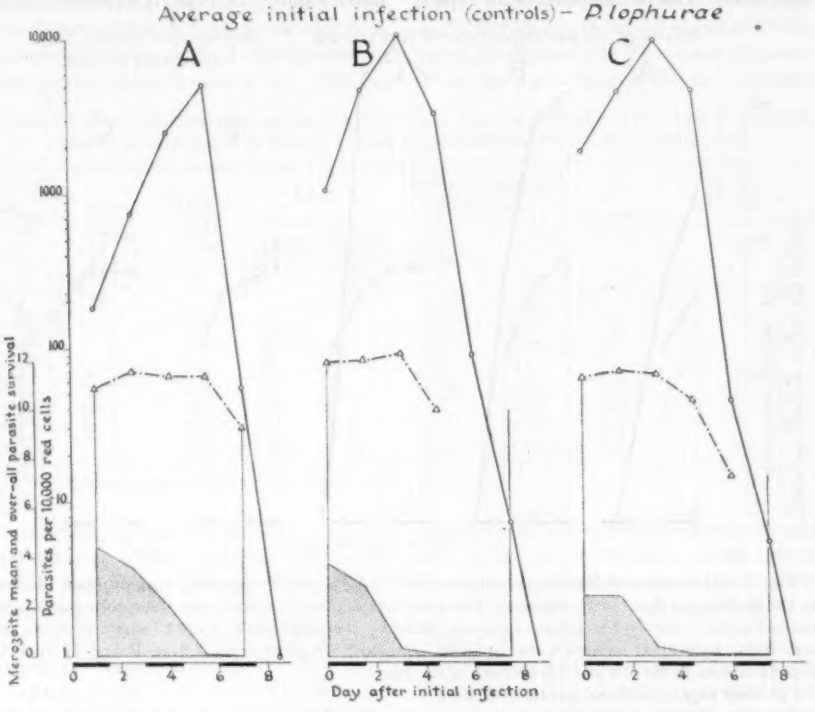

FIG. 9.—Controls for figure 8: Blood-induced initial infections of *P. lophurae* with inoculums comparable to those given type A, B and C superinfections. The parasitemia, rate of reproduction (merozoite mean) and over-all parasite survival and death rates are shown by averages from 7, 6 and 4 infections, respectively.

For other explanations, see legend in figure 2.

the three types of superinfections illustrated in figures 8 and 10 may be found in columns 8 through 10 in table 3. As contrasted with all types of superinfections of *P. lophurae*, control initial infections invariably showed increases of 1.3 through 4 (occasionally to 6) extracorpuscular merozoites at two or three segmentations (fig. 10, initial).

DISCUSSION

We have previously (1945) compared the course of initial infections of *P. gallinaceum* and *P. lophurae* and have studied the intensity of homologous and heterologous acquired immunity in both blood- and sporozoite-induced infections by means of blood-induced superinfections for both species and sporozoite-induced superinfections for *P. gallinaceum*. The present work is limited to blood-induced initial infections and homologous superinfections. It supplements the previous work in that initial infections of more variable intensity and duration were tested. Chiefly, however, it adds (1) a detailed consideration of the basic rate of parasite reproduction during initial infection and superinfection as determined by the mean number of merozoites produced by segmenters and the length of the asexual cycle, (2) a study of changes in the rate of reproduction and rate of survival of the parasites as initial infection and superinfection progress, and (3) the association of the admitted parasiticidal effects during immunity to superinfection with much of the inhibition of reproduction of the parasites.

As may be seen in table 2, fewer merozoites per segmenter were formed by *P. lophurae* (4 to 18) than by *P. gallinaceum* (4 to 40). Segmenters with an odd number of merozoites undoubtedly occurred in both species but were scarce. The trend in the merozoite mean per segmenter throughout the course of initial infection was also different in the two species. In *P. gallinaceum*, the mean was usually high (20 to 26) during the first through the third segmentation, decreased to a low value (16 to 22) at the parasite decline, increased (20 to 24) if the chicken did not die, and sometimes fluctuated thereafter. In *P. lophurae*, the merozoite mean was more or less constant during the acute rise and developed infection (10 to 13) and only decreased temporarily during the parasite decline (9 or less). These data during initial infection of *P. gallinaceum* corroborate and extend the findings of W. H. and L. G. Taliaferro (1948 and 1949b) and are essentially similar to those reported for *P. cathemerium* (Boyd and Allen, 1934, and Boyd, 1939), for *P. cynomolgi* (W. H. and L. G. Taliaferro, 1947), and for *P. knowlesi* (W. H. and L. G. Taliaferro, 1949a). Those of *P. lophurae* corroborate and extend the descriptions of W. H. and L. G. Taliaferro (1948) and are in general similar to those reported for *P. brasilianum* (W. H. and L. G. Taliaferro, 1944) and for *P. floridense* (Thompson, 1944).

Low merozoite means during the parasite decline were accompanied by a larger proportion of more markedly abnormal crisis forms in *P. gallinaceum* than in *P. lophurae* infections. The association of these two conditions in the various malarias we have studied leaves little doubt that both result from damage by fundamentally parasiticidal mechanisms.

Serial determinations of the merozoite mean during a given asexual cycle in infections of *P. gallinaceum* indicated that the merozoite mean was highest at maximal segmentation and then decreased to a value often lower than that encountered at maximal segmentation of the next asexual cycle. These decreases represented values of 10 to 15%. The merozoite means of major and minor broods varied similarly in infections of *P. brasilianum*, but were more

or less alike in infections of *P. lophurae* and *P. cynomolgi*. With respect to changes in the merozoite mean throughout infection, on the other hand, *P. gallinaceum* resembled *P. cynomolgi* and *P. lophurae* resembled *P. brasilianum*.

cycle together with decreases in the merozoite mean amounted to temporary decreases in reproduction of about 20 to 50%. This conclusion has already been reached for *P. gallinaceum* by W. H. and L. G. Taliaferro (1949b).

Over-all parasite survival rates were

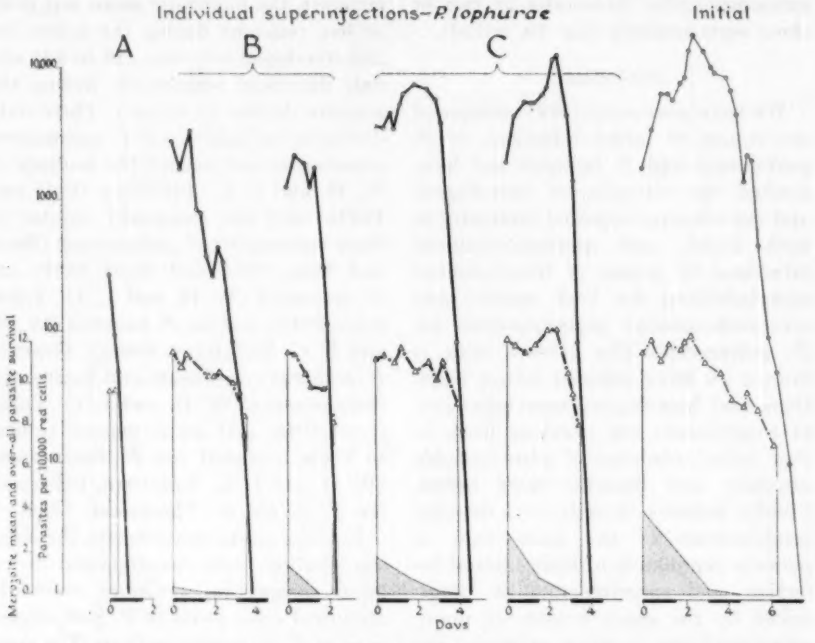

FIG. 10.—Homologous type A, B and C superinfections of *P. lophurae* from 5 chickens and of one control initial infection receiving the type B amount of inoculum.

For other explanations, see legend in figure 2.

Note that the parasitemia curves in type B and C superinfections increase periodically due to free merozoite survival at segmentation. The increases are not commensurate with the actual number produced.

The 36-hour asexual cycle with maximal segmentation occurring just after midnight one day and just after noon one and a half days later was not very synchronous in either species but was more synchronous in infections of *P. gallinaceum* than of *P. lophurae*. It was often prolonged in part or in whole at or near the crisis with proportionate realignments in the time of segmentation of the broods. Such delays in the asexual

higher at the first patent asexual cycle of *P. gallinaceum* (33 to 100 %) than of *P. lophurae* (20 to 60 %) infections. Stated otherwise, over-all parasite death rates due to innate immunity amounted to 0 to 66% for *P. gallinaceum* and 40 to 80 per cent for *P. lophurae*. The over-all death rates rapidly increased to 95% or more in *P. gallinaceum* infections but only increased to the same extent after having remained more or less constant

during the acute rise in *P. lophurae* infections.

Changes in the reproductive rate of these parasites during the course of the infection are almost certainly due to changes in the host rather than to any innate "developmental cycle" in the parasites (cf. Stauber, 1939). Thus, they are due to innate and/or acquired immunity. Recently, Trager and McGhee (1950) studied innate immunity (=natural resistance) against *P. lophurae*. They found a lessened parasitemia and a 5 to 10% decrease in the merozoite mean in chicks and ducklings injected with homologous normal hen and certain normal duck plasmas, respectively. In our own studies on *P. brasilianum*, we adduced evidence that they were largely associated with the specific parasiticidal factors of acquired immunity. These, although fundamentally parasiticidal, might inhibit reproduction by a generalized suppression of metabolic activities, including those of growth and reproduction. Such a condition is seen in immunity to *Nippostrongylus muris* and is markedly different from the specific inhibition of reproduction produced by ablastin in infections of *Trypanosoma lewisi* (see review in W. H. Taliaferro, 1948).

The question then arises: why does the parasiticidal activity remain long after the crisis when the rate of reproduction of the parasites has returned to normal or almost normal? We know nothing about the possible indirect effects through physiological derangements of the host produced by the toxicity of the antigen-antibody reaction suggested in our work on *P. brasilianum*. In final analysis, however, there may be no contradiction between a continuing parasiticidal action and lessened reproduction-inhibiting action. In fact, this condition may largely result from an increase in parasiticidal immunity. Thus, the occurrence of degenerate (crisis) forms and slower growth (lengthening of the asexual cycle and lower merozoite mean per segmenter) may occur at or near the crisis because of the presence of a large amount of antigen and/or a comparatively low titer of fundamentally parasiticidal antibodies which permits damaged parasites to survive long enough to be studied. During this period, small but varying proportions of the parasites are entirely normal in appearance. Later, as the titer of the parasiticidal antibodies increase, the susceptible parasites may quickly die and be removed, whereas the few parasites that live are entirely normal in their cytology, merozoite mean and length of cycle. It would seem that the progeny in these broods vary phenotypically and not genotypically in their susceptibility to antibodies, as otherwise the proportion of resistant parasites would quickly increase and repopulate the blood.

Homologous immunity to superinfection was essentially similar in both species except that it was just as strong at 5 and 6 months after an initial infection of *P. gallinaceum* as it was at 2 months and was definitely weaker 8½ months after an initial infection of *P. lophurae* than at 1 to 2 months. It behaved as does the initial infection beginning at the parasite decline or crisis in that (1) the merozoite mean was smaller (cf. table 2 and the work on *P. cathemerium* by Boyd and Gilkerson, 1942), (2) the asexual cycle was delayed to varying extents, (3) parasites were rapidly swept out of the blood, and (4) the parasiticidal effect of immunity was markedly stronger than the reproduction-inhibiting effect. With regard to this fourth effect, it is interesting that quinine exerted a stronger reproduction-inhibiting than parasiticidal effect against *P. gallinaceum* (cf. W. H. and L. G. Taliaferro, 1949b) and that nitrogen mustard affected *P. lophurae* in both capacities more adversely than *P.*

gallinaceum (W. H. and L. G. Taliaferro, 1948).

CONCLUSIONS

Initial infections of *Plasmodium gallinaceum* and *Plasmodium lophurae* have the same length asexual cycle (36 hours), but differ markedly with respect to synchronism of the cycle, rate of reproduction and survival and death of parasites. *P. gallinaceum* more nearly resembles *Plasmodium cynomolgi* and especially *Plasmodium knowlesi* although it forms more progeny. *P. lophurae* resembles *Plasmodium brasilianum*. Evidences of acquired immunity appeared progressively against *P. gallinaceum* and suddenly at the peak of the infection against *P. lophurae*.

Homologous immunity to superinfection in chickens was mainly parasiticidal against both *P. gallinaceum* and *P. lophurae* and acted on both free merozoites and intracorpuscular erythrocytic stages. To a less extent it inhibited reproduction by reducing the merozoite mean per segmenter and prolonging the asexual cycle. Thus, 97 to 99% of a superinfecting population was often destroyed within 36 hours, while reproduction was only temporarily decreased 10 to 50 %.

When optimal numbers of parasites with a normal merozoite mean and asexual cycle were introduced into latently infected chickens, the merozoite mean decreased sooner and reached a lower value, crisis forms appeared sooner and growth was sometimes slightly slower (lengthening of the asexual cycle) than in initial infections. These reproduction-inhibiting effects, which were not apparent when the superinfecting dose was large enough to break down acquired immunity, are apparently associated with fundamentally parasiticidal mechanisms (antibodies). After the acquisition of a high grade immunity as after the crisis, the few surviving parasites of each brood are apparently resistant to both parasiticidal and reproduction-inhibiting mechanisms, whereas the nonresistant parasites of each brood are removed from the blood before observations can be made on degeneration (as evidenced by crisis forms, lowered merozoite mean and lengthening of the asexual cycle).

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MORPHOLOGICAL OBSERVATIONS BY ELECTRON MICROSCOPY OF THE BRUNHILDE STRAIN OF POLIOMYELITIS VIRUS

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The strain of poliomyelitis virus used for this study, called the Brunhilde strain, was furnished by Dr. Howard A. Howe of the Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Maryland. It was isolated by him in 1939. The particular pool used, Brunhilde II, has a 50% end point of $10^{-5.8}$ in rhesus monkeys by intracerebral inoculation.

Twelve rhesus monkeys were inoculated intracerebrally with 1 ml of a 10^{-3} dilution of this poliomyelitis virus. The animals were sacrificed upon showing poliomyelitis symptoms. The spinal cords were removed aseptically and stored in a solid carbon dioxide chest at −40 C. A normal rhesus monkey was also sacrificed and the spinal cord removed asceptically and stored under the same conditions.

One week later 2 infected monkey spinal cords and a normal monkey spinal cord were removed from storage and were thawed at room temperature, then ground with alundum, and diluted to a 10% suspension with physiological saline. The 3 suspensions were then subjected to 5 minutes' centrifugation at 1000 r.p.m. in an angle centrifuge. The supernates were removed from all 3 specimens and filtered through a type ST size L3 Seitz filter. The filtrates from all 3 suspensions were then subjected to centrifugation in a Spinco Ultracentrifuge for 6 hours at 50,000 r.p.m. under refrigeration. The temperature of the refrigerated outer jacket stayed constant during the centrifuge run at −14 C. After the 6 hour centrifugation period was up, the supernates from the specimens were discarded and the sediment from each specimen was resuspended in 1 ml of normal physiological saline.

A small drop of each suspension was placed on parlodion film supports. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium[1] at arc tan $\frac{1}{8}$ and examined under the RCA electron microscope, type EMU. The balance of the infected material which was not used for the studies was injected intracerebrally into a rhesus monkey. The monkey showed typical poliomyelitis symptoms after a period of 6 days and became moribund on the 8th day. At that time it was sacrificed. Histological sections of the cord and brain showed typical pathological lesions of poliomyelitis.

Upon examination of the concentrated suspension of the normal spinal cord under the electron microscope, no virus-like particles could be demonstrated. Upon examination of the concentrated virus suspension virus-like particles were demonstrated as shown in figure 1. Some of the bodies are slightly spherical and slightly asymmetrical ranging in size from 20 mμ to 50 mμ. They somewhat resemble the bodies of the Lansing strain of polio-

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myelitis as described by Loring and coworkers.[2] In the electron examination a few filamentous forms were noted and one filamentous form is shown in figure 1.

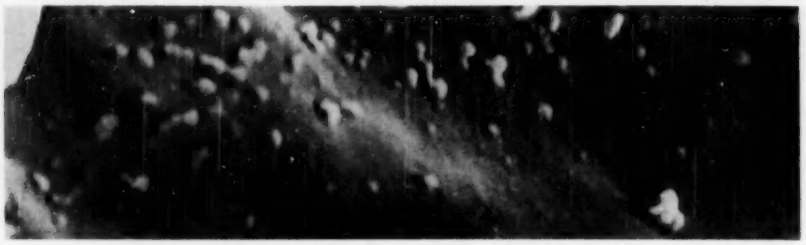

FIG. 1.—Poliomyelitis virus particles (Brunhilde strain) shadowed with chromium at arc tan $\frac{1}{8}$. ×85,400.

SUMMARY

Studies by electron microscopy of the spinal cord of monkeys infected with poliomyelitis virus (Brunhilde strain) shows the virus to be slightly spherical and slightly asymmetrical, ranging in size from 20 mμ to 50 mμ. A few filamentous forms were demonstrated. These bodies could not be demonstrated in concentrated normal monkey spinal cord subjected to the same procedure of concentration and electron examination. A monkey injected intracerebrally with this concentrated material showed poliomyelitis symptoms on the 6th day following injection.

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